AD_____

Award Number: DAMD17-96-1-6164

TITLE: The Role of a Novel Protease NES1 in Breast Cancer

PRINCIPAL INVESTIGATOR: Vimla Band, Ph.D.

CONTRACTING ORGANIZATION: New England Medical Center
Boston, Massachusetts 02111

REPORT DATE: April 2001

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 2001	Final (19 Aug 1996 - 18 Mar 2001

4. TITLE AND SUBTITLE
The Role of a Novel Protease NES1 in Breast Cancer

5. FUNDING NUMBERS
DAMD17-96-1-6164

6. AUTHOR(S)
Vimla Band, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
New England Medical Center
Boston, Massachusetts 02111

E-Mail:vband@lifespan.org

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

NES1 gene is expressed in normal mammary epithelial (MECs) and prostate cells, but its mRNA and protein expression is dramatically decreased in most established breast, prostate and cervical cancer cell lines even though no major deletion or rearrangements in NES1 gene are found. Inspite of high homology of NES1 polypeptide to known serine proteases, it lacks any detectable protease activity. This is probably due to the unusual amino terminus of NES1 that contains LDPEAY instead of IVGG (present in most active serine proteases). IVGG sequence is required for salt bridge formation. Transfection of NES1 cDNA into NES1-negative breast cancer cell line suppressed the anchorage-independent growth and tumor formation in nude mice. NES1 gene is localized on to chromosome 19q13.3. Cloning of NES1 promoter and analyses of its activity showed that most tumor cell lines were able to support full or partial transcription from NES1 promoter. We show that hypermethylation of NES1 gene on exon 3 is responsible for lack of NES1 expression in tumor cell lines and in primary breast tumors. In summary, we accomplished most of the tasks in Aim I, II and III. In addition, we defined the mechanism of loss of NES1 expression in tumor cells. Future studies should focus on analyses to evaluate NES1 expression/hypermethylation as potential marker for diagnosis and/or prognosis of breast and other cancers.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer, Mammary epithelial cells, Transformation, Protease, Radiation, Tumor Suppressors, Immortalization	64
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Liu X-L, Wazer DE, Watanabe K, and Band V. Identification of a novel serine protease-like gene, the expression of which is down-regulated during breast cancer progression. Cancer Res. 56:3371-3379, 1996.

Goyal J, Smith KM, Cowan JM, Wazer DE, Lee SW, and Band V. The role for NES1 serine protease as a novel tumor suppressor. Cancer Res. 58:4782-4786, 1998.

Summary of the original grant

The causative factors of breast cancer are largely unknown. A role for radiation is suggested by higher incidence of breast cancer following diagnostic or therapeutic radiation and in survivors of atomic bombing in Japan. However, little is known about the molecular basis of radiation-induced transformation of mammary epithelial cells (MECs). We have derived a radiation-transformed MEC, 76R-30, by fractionated irradiation of a normal MEC strain 76N. We prepared a cDNA library from 76N cells in which 76R-30 cross-hybridizing mRNA species were depleted by subtractive hybridization. Differential screening of this library with 76N and 76R-30 cDNA probes led to identification of a novel gene, NES1, whose mRNA expression was drastically reduced in 76R-30 cells. Importantly, NES1 mRNA expression was lost in the majority of breast cancer cell lines, but was high in normal and immortalized (non-tumorigenic) MECs. NES1 mRNA levels were markedly reduced when immortalized breast cells were tumorigenically transformed with activated H-ras oncogene. Significantly, NES1 expression decreased after chronic treatment of MECs with gamma-irradiation. NES1 mRNA expression was cell cycle regulated. Sequence comparison of full length NES1 cDNA revealed it to be a novel member of the serine protease family.

Here, we propose to generate anti-NES1 antibodies, and examine the expression of NES1 in normal and tumor mammary tissue sections and in cultured cells. We will examine the expression of NES1 mRNA and protein in response to gamma-irradiation, DNA damage and cell growth/differentiation agents, and examine the mechanisms of this regulation. In order to assess the role of NES1 in MECs, we will overexpress NES1 and assess its effect on growth and tumorigenic properties. Alternatively, we will attempt to decrease or eliminate NES1 expression and determine if this results in increased growth, lengthening of life span, immortalization or tumorigenicity. Finally, we will demonstrate the protease activity and recognition specificity of NES1 and attempt to identify the potential cellular targets of NES1 protease activity. Together, these studies should reveal the functional role of a novel serine protease whose expression is down-regulated during radiation-induced transformation of mammary cells. Thus, these studies should directly contribute to further our understanding of the biology of radiation-induced and other forms of breast cancer.

SPECIFIC AIMS OF THE ORIGINAL GRANT

I. **CHARACTERIZE THE EXPRESSION OF NES1 IN NORMAL AND TUMOR BREAST CELLS IN CULTURE AND IN TISSUE SPECIMENS.**
   1. Generate anti-NES1 antibodies.
   2. Examine the expression of NES1 mRNA and protein in normal and tumor mammary cells in tissue sections and in cultures.
   3. Examine the mechanisms of inducible NES1 expression.
   4. Examine the effect of DNA damage on NES1 mRNA and protein expression.

II. **ASSESS THE INFLUENCE OF ALTERATIONS IN NES1 EXPRESSION ON CELL GROWTH AND ONCOGENICITY.**

1. Transfection of NES1 into mammary cells.
2. Growth properties and oncogenic behavior of NES1 transfectants.
3. Influence of reduced NES1 expression on growth, immortalization and oncogenicity of MECs.

III. **CHARACTERIZE THE BIOCHEMICAL FUNCTIONS OF NES1.**

1. Characterize the potential protease activity of NES1.
2. Examine non-protease biochemical functions of NES1 protein.
3. Mutational analysis of NES1 protein.

INTRODUCTION:

Breast cancer is the second leading cause of cancer-related deaths in the United States. Although a number of genetic markers have been identified for hereditary breast cancer in recent years, much less progress has been made in defining molecular markers for sporadic non-hereditary breast cancer, which account for the vast majority of breast cancers. Using subtractive hybridization between a radiation-transformed breast epithelial cell line and its isogenic normal parental cell strain, 76N, we previously identified a gene NES1 (Normal Epithelial Specific 1) that was expressed in normal but not in radiation-transformed MECs (See appendix Ms. 1, Liu et al., 1996). Significantly, NES1 mRNA was either absent or drastically reduced in a majority of established breast cancer cell lines. Complete sequencing of multiple NES1 cDNA clones revealed it to be a previously unknown protein with significant homology to trypsin-like serine proteases (Appendix Ms. # 1). We hypothesized that NES1 is involved in maintenance of the untransformed state of mammary epithelial cells and elimination of its expression or function may constitute one of the steps in the multi-step tumorigenesis. The essence of this grant was to examine the structure, expression and functional role of NES1 in the control of mammary epithelial cell growth and oncogenesis.

BODY OF THE REPORT

Tissue-specific expression of NES1 mRNA (Aim I-2) [See appendix Ms. # 1].

To assess the tissue distribution of NES1 expression, poly(A)+RNA samples from various human tissues (Nylon purchased from Clontech) were hybridized with a NES1 probe corresponding to nucleotides 1-1069 of the full-length cDNA. As shown in Fig. 1, differential expression of the NES1 gene was observed in the tissues examined. Relatively abundant levels of the 1.4 kb mRNA transcript were observed in the prostate, testis, ovary, small intestine, colon, and lung with highest levels in the ovary. The pancreas showed abundant expression of a shorter (1.1 kb) mRNA transcript. In comparison, NES1 expression in thymus and heart was barely detectable and was essentially undetectable in peripheral blood leukocytes, brain, placenta, lung, liver, skeletal muscle and kidney. Hybridization with the control probe 36B4 demonstrated equal loading of mRNA in all lanes. These analyses show that NES1 mRNA is expressed in a large subset of human tissues.

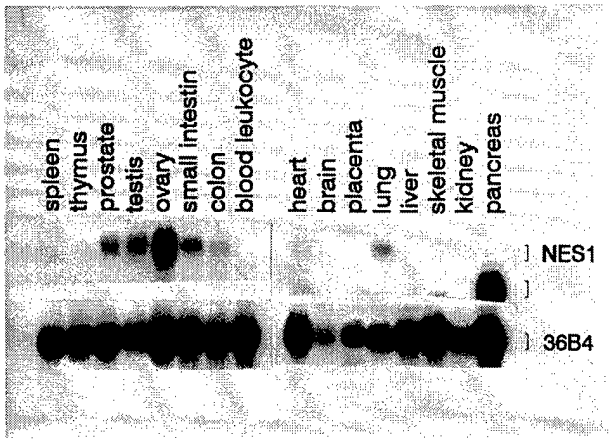


Fig. 1. Northern blot analysis of NES1 mRNA expression in various human tissues. Nylon membranes with 2 μg of poly(A)+ RNAs from each of the indicated human tissues were obtained from Clontech, hybridized with a 32P-labelled NES1 probe and visualized by autoradiography. 36B4 probe was used as a loading control.

Expression of NES1 mRNA in normal, immortalized and tumor mammary cells (Aim I-2) [see Appendix Ms. # 1].

As described in appendix Ms. #1, NES1 cDNA was isolated by virtue of its drastically reduced mRNA expression in MECs oncogenically transformed by γ-irradiation. To further assess the relationship between NES1 mRNA expression and mammary tumor progression, we analyzed a number of normal MECs, mammary fibroblasts, immortalized MECs and established mammary tumor cell lines by Northern blot analysis. All of the normal (4 out of 4) mammary epithelial cells expressed abundant levels of 1.4 kb NES1 mRNA, whereas none of the fibroblast cell strains (5 out of 5) expressed detectable mRNA levels (hence the designation of Normal Epithelial Cell Specific-1 or NES-1) (Fig. 2 and data not shown). All of the MECs that have been induced to undergo pre-neoplastic transformation by human papilloma virus (HPV)-16 E6 or E7 (10 out of 10) expressed NES1 mRNA levels similar to normal MECs (Fig. 2 and data not shown). In contrast, a drastic decrease or complete loss of NES1 mRNA was observed in all (16 out of 16) breast cancer cell lines examined (Fig. 2). Reprobing of the blot with a control probe 36B4 showed that mRNA was indeed loaded in the lanes that showed little or no hybridization with NES1 probe (Fig. 2). These results show that while NES1 mRNA expression is unaffected by immortalization (preneoplastic transformation) of mammary cells, it is drastically down-regulated in a large subset of established breast cancer cell lines.

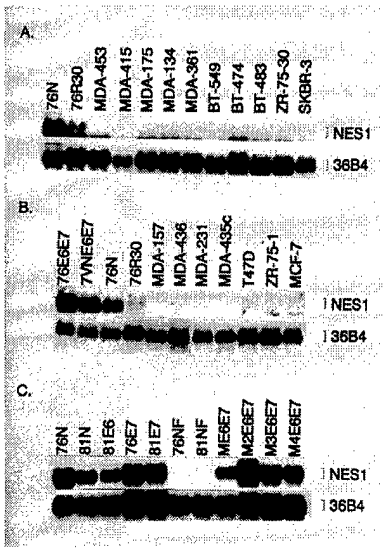


Fig. 2. Northern blot analysis of NES1 mRNA expression in cultured cell lines. Total cellular RNA (10 μg) from various cells was resolved on a 1.5 % agarose-formaldehyde gel, transferred to nylon membrane, and hybridized with a 0.4-kb NES1 probe. Note a drastic decrease in NES1 mRNA levels in 76R-30 cells and almost complete loss in all mammary tumor cell lines. 36B4 was used as a loading control. A, B, and C are separate blots. A. 76N, normal MEC; 76R-30, radiation-transformed 76N; remaining cell lines represent breast cancer-derived cell lines. B. 76E6E7, 76N cells immortalized by HPV-16 E6+E7; 7VNE6E7, 7VN cells immortalized by HPV-16 E6+E7; 76N and 76R-30 as in A; remaining cell lines represent breast cancer-derived cell lines. C. First two lanes, 76N and 81N are two normal mammary epithelial cell strains; 81E6, a HPV-16 E6-immortalized 81N cells; 76E7 and 81E7 are HPV-16 E7-immortalized 76N and 81N cells respectively; 76NF and 81NF are two mammary fibroblast cell strains from two individuals designated 76 and 81 respectively; rest four lanes are HPV-16 E6+E7-immortalized human milk derived epithelial cell lines from three separate specimens.

Generation of anti-NES1 peptide antibody (Aim I-1).

Two NES1 peptides (a.a. 120-137, n.C-K-Y-H-Q-G-S-G-P-I-L-P-R-R-T-D-E-H-D.c; a.a. 38-50, n.Q-N-D-T-R-L-D-P-E-A-Y-G-A-P-C...c) were coupled to Keyhole limpet hemocyanin through a C-terminal cysteine residue using maleimidobenzoyl-N-hydroxysuccinimide ester (Pierce, Rockford, IL) for chemical cross-linking. The conjugates were used to immunize rabbits as a 1:1 emulsion with Titermax adjuvant (CYTRX Corporation, Georgia). Booster injections were given every 30 days and rabbits were bled 10 days after each injection. As shown below in Fig. 3, anti-peptide antibody (a.a 120-137) recognizes NES1 protein by Western blot analysis. Similar results were obtained with second anti-peptide antibody (see Fig. 4).

NES1 is a secreted protein (Aim I-2).

Most of the proteins that showed homology to predicted NES1 polypeptide contain a signal peptide at the N-terminus (first 14 to 15 amino acids after methionine) and are secreted, although the N-terminus of NES1 did not show a significant homology to corresponding region of the other serine proteases. This region was strongly hydrophobic (see appendix manuscript # 1), consistent with its being a signal peptide and suggestive of NES1 being a secreted protein. To examine this possibility, we used a rabbit antiserum against a NES1-specific peptide (a.a. 120-137). This antiserum was used for Western blot analysis of cell lysates and culture supernatants derived from NES1 mRNA-positive and -negative MECs (Fig. 2). As seen in Fig. 3, anti-NES1 antibodies specifically detected a 30 kDa polypeptide almost exclusively in the culture supernatants of all of the NES1 mRNA-positive cell lines. The 30 kDa polypeptide was not detected in the NES1 mRNA-negative cell lines. These results demonstrate that NES1 is a secreted protein similar to other homologous serine proteases.

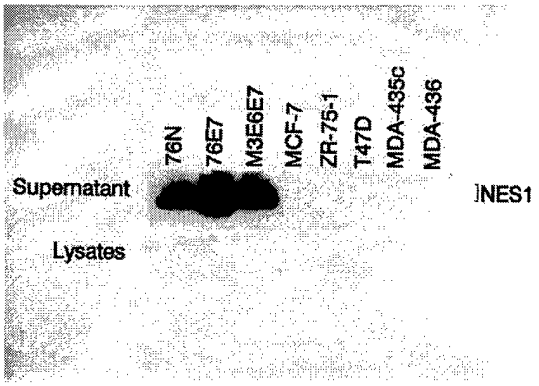


Fig. 3. Western blot analysis of NES1 protein. Aliquots of concentrated culture supernatant or cell lysates derived from a number of indicated cell lines were resolved by a SDS 10 %-PAGE and transferred to PVDF membrane. Membranes were immunoblotted with anti-NES1 antiserum followed by goat anti-rabbit IgG conjugated to horse-radish peroxidase. Detection was by enhanced chemiluminescence.

Expression of NES1 in an NES1-negative breast cancer cell line MDA-MB-231 results in reduced anchorage-independent growth and tumor formation in nude mouse (Aim II-2) [see Appendix Ms. # 2]. The pattern of NES1 expression, with high levels in normal and immortal mammary epithelial cells, reduced levels in radiation-transformed 76R-30 cells and an essentially complete lack of expression in most mammary tumor cell lines, suggested a potential role of NES1 in tumor suppression. To directly assess if NES1 can function as a tumor suppressor protein, we introduced either the pCMVneo vector or NES1-pCMVneo plasmids into a breast cancer cell line, MDA-MB-231. The choice of MDA-MB-231 cell line was based on its lack of NES1 expression, its known ability to grow in an anchorage independent manner and its ability to form tumors when implanted in nude mouse. Following G418 selection, six independent stable clones each of the vector- and the NES1-transfected MDA-MB-231 cells were tested for the expression of NES1 mRNA and protein. As shown in Fig. 4 A, 4 out of 6 NES1-transfected MDA-MB-231 clones (clone #s 2, 4, 5, and 6) expressed high levels of NES1

mRNA while the remaining two clones (clone #1, and 3) showed very little or no mRNA expression. When analyzed for NES1 protein, the four high mRNA-positive clones (clone # 2, 4, 5, and 6) showed considerably high levels of protein as compared to low mRNA-expressing clones (clone # 1, and 3) (Fig. 4 B). As expected, none of the vector-transfectants showed any NES1 protein (lane 1 and data not shown). Based on the protein expression, the three strong positive clones (2, 4, and 5) were used for further analyses to examine the effect of NES1 on tumorigenicity.

Previous studies have demonstrated a direct correlation between the tumorigenic phenotype of cancer cell lines, such as MDA-MB-231, and the ability to grow in a anchorage-independent manner and to form tumors when implanted in nude mice. As shown in Fig. 5 A, each of the three vector-transfected clones gave rise to colonies when seeded in soft agar, while the clonogenicity of the three NES1-transfectants was markedly decreased. The experiment was repeated three times, each in triplicate, with similar results. Thus, overexpression of NES1 in a NES1-negative breast cancer cell line abolished the ability of cells to grow in an anchorage-independent manner.

When $5x10^5$ cells of NES1-transfectant clone # 4 or a vector-transfectant were injected subcutaneously into the mammary gland area of mice, 5 out of 5 vector-transfected mice showed palpable tumors within 8-10 days of injection and these grew progressively reaching a 2.0 x 2.0 cm^2 size by 4 weeks (Fig. 5 B). The tumor from one vector-transfectant implanted mouse was excised for histopathology and to assess the ability of tumor cells to grow in vitro. These experiments demonstrated that the tumor was an adenocarcinoma, and these cells proliferated in cell culture (data not shown). In contrast to vector-transfected MDA-MB-231 cells, none of the mice implanted with NES1-transfected MDA-MB-231 cells showed any palpable or visible tumors by 4 weeks. Mice were then sacrificed and the injected area was examined thoroughly for any non-palpable tumor growth. However, no tumor growth was observed. Taken together, these data clearly demonstrate the ability of NES1 to suppress the tumorigenicity in MDA-MB-231 cells.

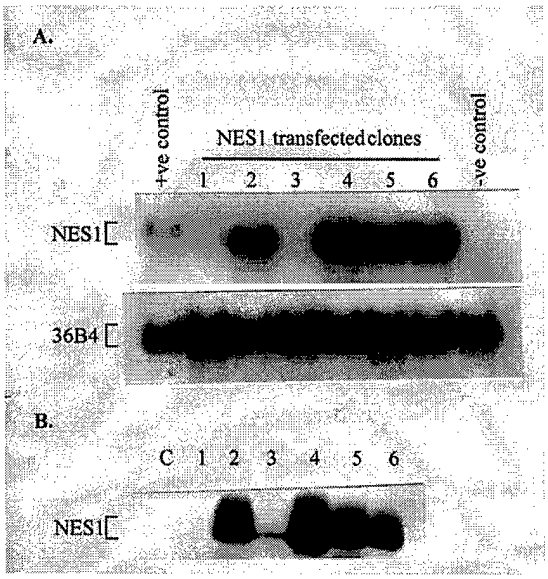


Fig. 4 A. Northern blot analysis of NES1 mRNA expression in NES1-transfected MDA-MB-231 cells. Nylon membranes with 10 μg of total RNA from different NES1-transfected clones (1-6) of MDA-MB-231 cells were hybridized with a ^{32}P-labelled NES1 probe and visualized by autoradiography. Positive (+ve) control, normal mammary epithelial 76N cells; Negative (-ve) control, vector-transfected MDA-MB-231 cells. 36B4 probe was used as a loading control. **B. Western blot analysis of NES1 protein in NES1-transfected MDA-MB-231 cells.** Aliquots of culture supernatant derived from different NES1-transfected clones (1-6) of MDA-MB-231 cells containing 50 μg of protein were resolved by SDS-10 % PAGE and transferred to PVDF membrane. Membranes were immunoblotted with an anti-NES1 antiserum (against a.a. 38-50) followed by goat anti-rabbit IgG conjugated to horse-radish peroxidase. Detection was by enhanced chemiluminescence. Control (C), supernatant from vector-transfected MDA-MB-231 cells.

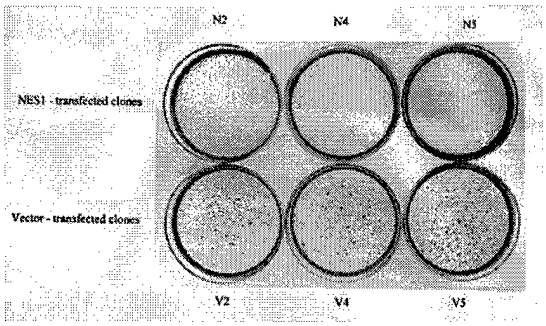


Fig. 5 A. Anchorage-independent growth of NES1-transfected clones. Various clones of vector-transfected (V2, V4, V5) or NES1-transfected (N2, N4, N5) MDA-MB-231 cells (1 X 10^5/60 mm dish) were plated in soft agar and colonies were photographed after 2 weeks.

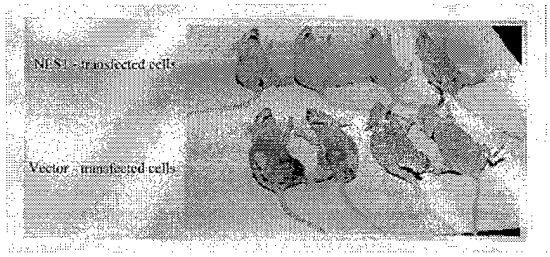
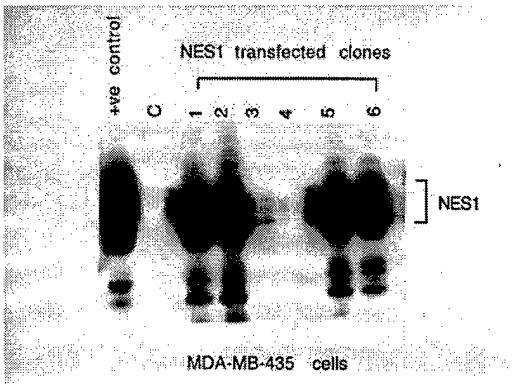
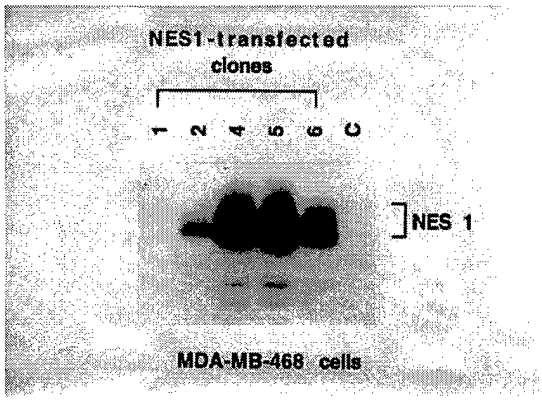


Fig. 5 B. Growth of vector- or NES1-transfected MDA-MB-231 cells as tumors upon implantation in nude mice. 5x10^5 cells of vector or NES1-transfected MDA-MB-231 cells were injected subcutaneously into the mammary fat pad area below the nipple. Tumors were allowed to grow for 4 weeks at which time the mice were photographed and sacrificed.

In view of the dramatic reduction in anchorage-independent growth of MDA-MB-231 cells upon NES1 overexpression, we transfected NES1 into two other breast cancer cell lines MDA-MB-435 and MDA-MB-468. Both of these cell lines are known to be highly tumorigenic when implanted in the mammary fat pad of the nude mice, and this would allow an assessment of the

effect of NES1 expression on tumorigenicity. Fig. 6 and 7 show NES1 protein expression in NES1 transfectants of MDA-MB-435 and MDA-MB-468 respectively. In each case, several clonal transfectants expressing NES1 mRNA and secreting NES1 protein into culture medium were obtained. These transfectants were analyzed in vitro in soft agar cloning assay and found to behave similarly as NES1-transfectants of MDA-MB-231 cells (Fig. 8 and data not shown).





Figs 6 and 7. Protein analysis in the supernatants of NES1-transfected clones of MDA-MB-435 and MDA-MB-468 by Western Blot Analysis. Control or NES1-transfectants were grown to 80-90% confluency in 25 cm^2 flasks in α-MEM containing FCS. Cells were then washed once with a serum-free medium and further cultured in this medium for 20 h. 50 μg of protein from unconcentrated supernatants were used to analyze in Western blot as described in Fig. 4. Fig. 6 shows 4 out of 6 clones (clone # 1, 2, 5 and 6) expressing high levels of NES1 protein in MDA-MB-435 cells. Fig. 7 shows 3 (clone # 4, 5, and 6) out of 5 clones expressing high levels of NES1 protein in MDA-MB-468 cells. All clones that show high levels of NES1 protein in Figs. 6 and 7 also showed high levels of mRNA (data not shown).

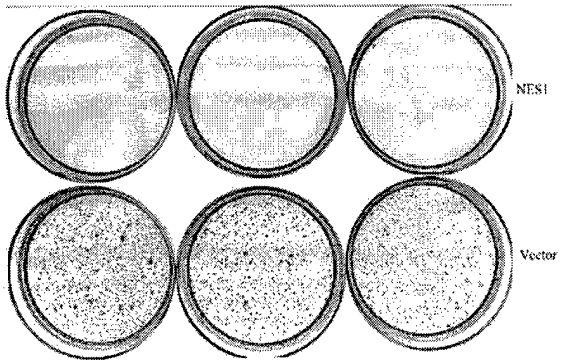


Fig. 8. Anchorage-independent growth of NES1-transfected clones in MDA-MB-435 cells. Various clones of vector-transfected (V2, V4, V5) or NES1-transfected (N2, N5, N6) MDA-MB-435 cells (1 X 10^5/60 mm dish) were plated in soft agar and colonies were photographed after 2 weeks.

Localization of the NES1 gene to chromosome 19q13.3 by Fluorescence In Situ Hybridization analysis (New task) [For Fig. Please see Appendix Ms. # 2]. The potential role of NES1 as a tumor suppressor, as suggested by the above experiments, prompted us to examine the chromosomal localization of the NES1 gene. For this purpose, we performed pulsed-field gel electrophoresis of a Not1-digested bacterial artificial chromosome (Bac) clone and found that it contained a 140 Kb NES1 DNA insert. Southern blotting of an EcoRI-digest of DNA isolated from several single colonies of this Bac clone, compared with genomic DNA of 76N normal mammary epithelial cell strain using a NES1 cDNA probe indicated that the 140 Kb genomic clone contained nearly all of the NES1 gene (data not shown). This 140 Kb probe was used for FISH analysis. Thirty one metaphase spreads were analyzed for chromosomal localization of NES1. In all metaphase spreads, hybridization signals were found on both copies of the 19q (See Appendix Ms. # 2). Longer metaphase spreads allowed localization of the NES1 gene to 19q13.3 and 90 % of these spreads had both chromatids stained. Propidium Iodide counter staining to evaluate chromosomal banding confirmed the presence of NES1 on 19q13.3 (data not shown). This analysis localizes the NES1 gene to the same region where the gene for prostate cancer-associated serine protease PSA is localized (See Appendix Ms. # 2).

NES1 mRNA and protein expression is down-regulated in prostate cancer cell lines (New task) [See Appendix Ms # 2]. The localization of NES1 gene to chromosome 19q13.3, where prostate specific antigen is localized, prompted us to analyze the status of NES1 expression in normal, immortal and tumor cells derived from the prostate gland. Northern blot analysis revealed that while NES1 was expressed at high levels in normal tissue from a prostactomy specimen, and two HPV-18 and SV40-immortalized prostate cell lines derived from normal prostate epithelial cells, four out of four prostate tumor cell lines completely lacked the NES1 mRNA expression (Fig. 9A). Importantly, the analysis of NES1 protein in supernatants revealed that the immortalized prostate cells secreted significant levels of NES1 protein whereas no protein was detected in the supernatants of tumorigenic prostate cell lines (Fig. 9 B). Importantly, when these supernatants were tested for PSA levels, a significant level of PSA protein was detected in one of the four (LNCaP) prostate cancer cell line. These data are

consistent with the published reports on the secreted levels of PSA in LNCaP cells. Thus, similar to our previous results in breast cells, NES1 mRNA expression is downregulated during tumorigenic progression in prostate cancer. Taken together, these experiments further suggest that NES1 may play a tumor suppressor role in breast cancer, as well as in prostate cancer.

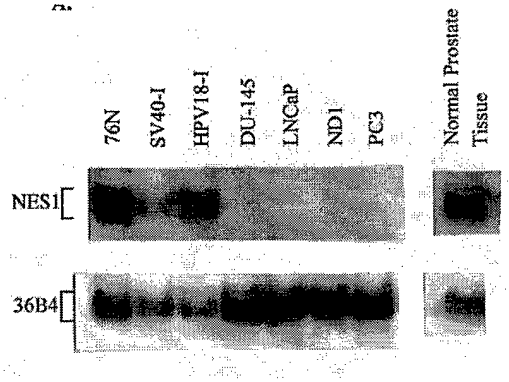


Fig. 9 A. Northern blot analysis of NES1 mRNA expression in prostate-derived normal, immortalized and tumor epithelial cells. Nylon membranes with 10 μg of total RNA from normal prostate tissue, immortalized prostate cell lines SV40-I and HPV18-I, and prostate tumor cell lines DU-145, LNCaP, ND1 and PC3, were hybridized with a ^{32}P-labelled NES1 probe and visualized by autoradiography. 76N normal mammary epithelial cell strain was used as a positive control. 36B4 probe was used as a loading control.

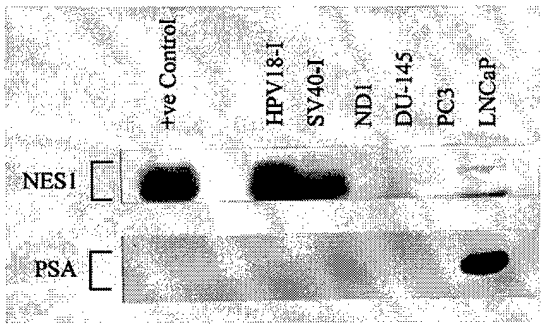


Fig. 9 B. Western blot analysis of NES1 protein in prostate-derived immortalized and tumor epithelial cell lines. Aliquots of culture supernatants derived from different cells (as indicated above) containing 300 μg protein were resolved by SDS-10 % PAGE and transferred to PVDF membrane. Membranes were immunoblotted either with an anti-NES1 antiserum (above panel) or with an anti-PSA monoclonal antibody followed by goat anti-rabbit IgG or goat anti-mouse IgG horse-radish peroxidase conjugates, respectively. Detection was by enhanced chemiluminescence. Positive (+ve) control, supernatant from NES1-transfected MDA-MB-231 cells.

NES1 inhibits branching morphogenesis of MDA-MB 231 cells (AIM II). Since NES1 has homology with serine protease and its overexpression inhibits soft agar growth as well as tumor formation, we analyzed the effect of overexpression of NES1 on cell spreading. For this, NES1 and vector transfected MDA-MB-231 cells were grown in reconstituted basement membrane, matrigel. Matrigel is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumor rich in extracellular matrix proteins. Its major components are laminin, collagen IV proteoglycans, TGFβ, fibroblast growth factor, tissue plasminogen activator and other growth factors. Matrigel is commonly used to recreate the *in vivo*-like tissue architectural phenotypes and has proven useful for studying morphogenesis of a number of epithelial tissue types, the most dramatic example being the assembly of structures resembling the alveoli of lactating mammary glands and secretion of milk proteins by mouse mammary epithelial cells when maintained in matrigel. Matrigel has also been used successfully to study invasive potential of cancer cells. Previous studies have correlated these morphological changes of cancer cells embedded in matrigel with their metastatic potential. Previous studies have shown invasive cells such as MDA-MB 231, 436, 435, BT549 and HS578T cells exhibit branching morphology in matrigel in contrast to non-invasive cells such as MCF-7 and T-47D that exhibit smooth spherical colonies on matrigel. Our hypothesis was that re-expression of NES1 in invasive MDA-MB-231 cancer cells may cause non-invasive normal phenotypic changes. Even though no morphological differences were noticed between vector and NES1 transfected cells when cultured on plastic, we wished to examine if NES1 inhibits the growth of MDA-MB-231 cells when grown on matrigel. As shown in Fig. 10, under these conditions, the two cell types showed striking differences. Vector-transfected cells extended, spread and formed branching structures, whereas NES1-transfected cells aggregated into tight smooth and spherical colonies (Fig. 10 A & B). This distinct behavior was apparent after 24-48 h in culture. Furthermore, the NES1-transfectants did not proliferate readily on the matrix, whereas the vector-transfected cells reached high density and covered the dish over the course of a week. These data clearly demonstrate that NES1 inhibits the invasive phenotype of breast cells.

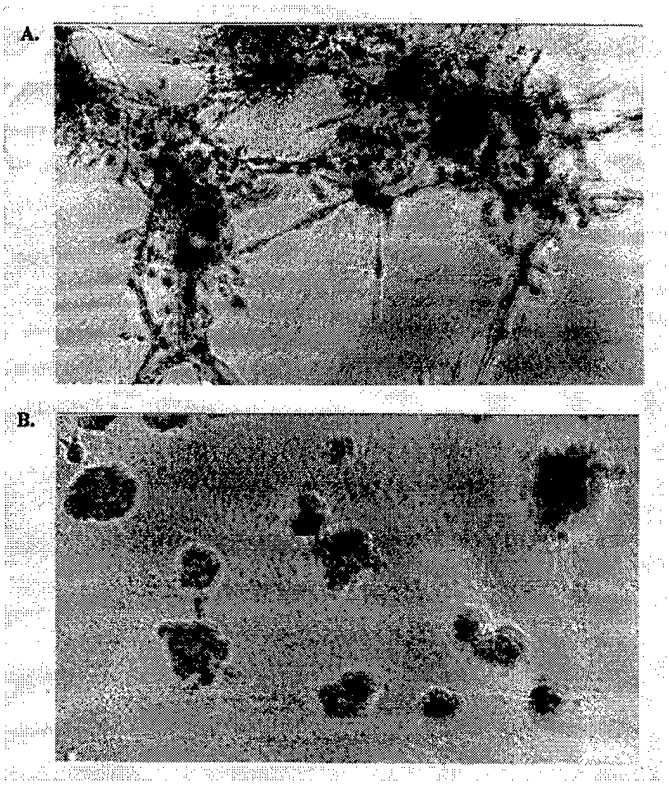


Fig. 10. NES1 overexpression inhibits morphological branching on matrigel. 1.5×10^4 vector-transfected (A) or NES1-transfected MDA-MB-231 cells were cultured on matrigel (1:1 diluted with serum-free medium) in 24 well plates. Cells were photographed after six days.

WORK DESCRIBED BELOW IS CURRENTLY UNDER PREPARATION. ONCE IN PRESS, WE WILL FORWARD PREPRINTS/REPRINTS TO DOD.

CpG METHYLATION IN EXON 3 AS A BASIS FOR BREAST TUMOR-SPECIFIC LOSS OF NES1 EXPRESSION

Characterization of NES1 mRNA expression in various breast and cervical cancer cell lines (Aim I). While we have previously demonstrated that NES1 mRNA is expressed in normal and immortal MECs and prostate cells, and reduced or completely absent in most breast and prostate cancer cell lines (appendix Ms. 1), the panel of cell lines used here included those not previously analyzed breast cells (76NTERT, 76E6, 70E6, 21PT) and cervical cancer cell lines (Siha, Caski and Hela). Therefore, we first examined the NES1 mRNA expression in the full panel of cell lines used in the study described below, by carrying out Northern Blot analysis. As expected, abundant levels of NES1 mRNA were observed in normal (76N, 81N) or immortal (76E6, 81E6, 70E6, 76NTERT) MECs. In contrast, NES1 expression was undetectable in majority of tumor cell lines (MDA-MB-231, MDA-MB-453, MDA-MB-435, MCF-7, Siha and Hela). However, one breast (21PT) and one cervical (Caski) cancer cell line expressed easily detectable levels of

NES1 mRNA (Fig. 11). Together, these cell lines provided a panel of NES1-positive and -negative cells for further analyses to define the mechanism of tumor-specific loss of NES1 expression.

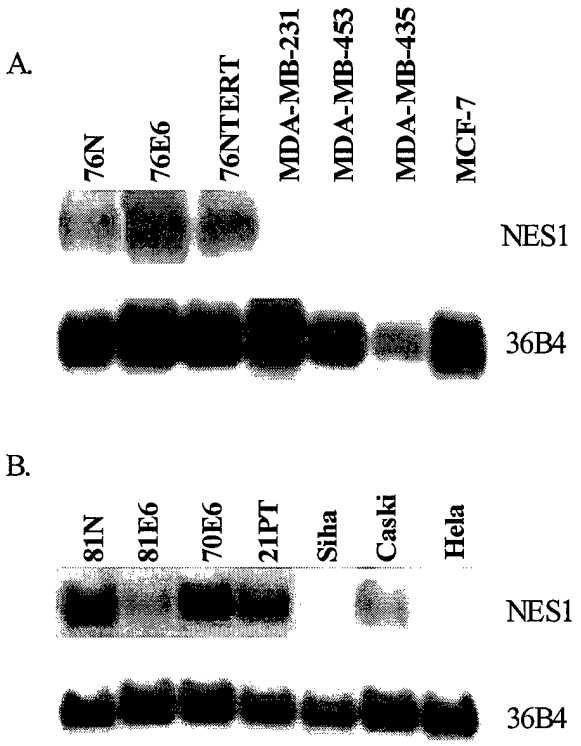


Fig. 11. Northern blot analysis of NES1 mRNA expression in cultured cell lines. Analysis was performed as described in Fig. 2 legend. 36B4 was used as a loading control.

Identification of the NES1 gene Promoter (New task). One potential mechanism for tumor-specific lack of NES1 mRNA expression is the inability of these cells to support NES1 transcription due to loss of critical transactivating factors or expression of repressor factors. In order to assess this possibility, we undertook the identification of the NES1 promoter. For this purpose, a NES1 cDNA was used as a probe to isolate a 140-kb human genomic DNA fragment from a bacterial artificial chromosome (Bac) library. Purified Bac DNA was digested with various restriction enzymes and hybridized with human NES1 cDNA (data not shown). These analyses allowed the isolation of a single 6.5 kb Hind III fragment that included exon 1 to 6 of the NES1 gene as well as a small region of untranslated region (Fig. 12 A). A 354 bp Hind III-Pst I fragment to corresponding to nucleotides -62 to +292 relative to first nucleotide of the 73 bp exon 1 was used as a probe to identify a single 3.1 kb BamH I fragment that included sequences -2875 to +247 relative to first nucleotide of exon 1 (data not shown); this clone included part of exon 2, intron 1 and the putative NES1 promoter (Fig. 12 A). Sequence of the promoter region is shown in Fig. 12 B and the promoter and exon/intron regions included in this fragment are schematically represented on top of Fig. 12 C. The cloned NES1 promoter lacks a consensus TATA box and contains potential AP1-binding, SP1-binding and CRE (cyclic AMP responsive element) sequences, consistent with the clustering of such sequences within the promoter region (Fig. 12 B).

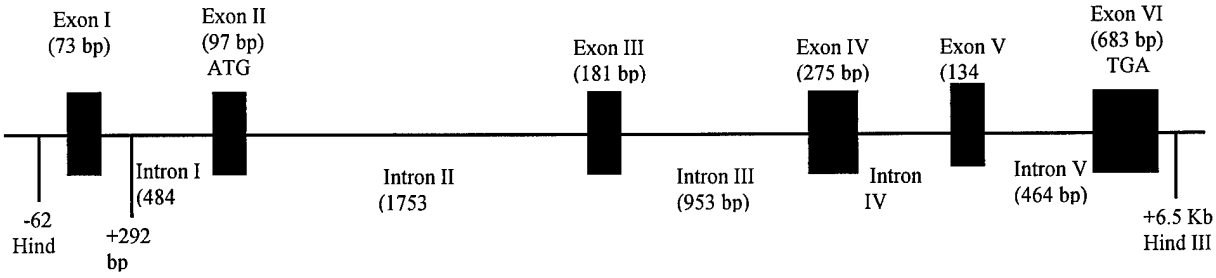


Fig. 12A. Genomic organization of NES1.
This schmatic diagram was drawn based on information published in Luo et al., Biochem. Biophy. Res. Commun. 247, 580-586, 1998.

-2875

GGATCCAGGAGACGATGAAGAACAATTAGACTGGACCCACCCACCACAGCCCATCACCCTCCATTTCCACTTGGTGTTTGGT
TCCTGTTCACTCTGTTAATAAGAAACCCTAAGCCAAGACCCTCTACGAACATTCTTTGGGCCTCCTGGACTACAGGAGATGC
TGTCACTTAATAATCAACCTGGGGTTCGAAATCAGTGAGACCTGGATTCAAATTCTGCCTTGAAATATTGTGACTCTGGGAA
TGACAACACCTGGTTTGTTCTCTGTTGTATCCCCAGCCCCAAAGACAGCTCCTGGCCATATATCAAGGTTTCAATAAATATT
TGCTAAATGAGTGAATCTACTGAGTGCTTACTATGTGCTAGACCCTGATCCAATGGCTTTTATTTTATTTTATTTTTTGACA
GAGTCTCGCTCTGTCACCCAGGCTGGAGTACAGTGGTGCTATCTCTGCTCACTGCAACCTCCACCTCCTGGGTTCAAGCAAT
TCTCCTGCCTCAGCCTCCTGAATAGCTGGGATTACAGGTGCCTACCACCACATCCGGCTAATTTTTGTATTTTTTAGTAGAG
ATGGGGCTTCACCATGTTGGCCAGGCTGGTCTCGAACTCCTGACCTCAGATGATCTGCCCTCCTTGGCCTCCCAAACTCCTG
GGATTACAGACGTGAGCCACCGCGCCCGCCCGGCTTTCATTTATTAATTAAAAGAAATTAAATTAATTAATCTATTTAGGAG
ACAGTCTTGCTCTGTTGCCCAGGCTGGAGTGCAGTAACAATCACAGCTCACGGCAATCTCAATTTCCTGGGGTCAAGTGATT
GTCCTCCCTCAGCCTCCAGAGTAGCTGGGACTACAGGCACATGCCACGAAGCCCAGCTAATTTTTGTATTTTTCGTAGAGAC
AGAGGTCTCAGTATGTTGCCCCGGCTAGTCTCAAACTCCTGGGCTCAAGCAGTCTGTCCTCCTCAGCCTCCAAAAGTGGTGA
GATTACAGGCATGAGTCGCTGTGCCTGGCCTCCAAGCACTTTCAAATGTATCAACTTAATCCTCACAAAACCCTGTGAGGTC
GGTACTGTTTTCATACCTATTTTATAGTTGAAGAAACAGACACAGAGAAGCAAAGTCACTTGCTCACAGTCACGTGGCTAGG
AGAGCAAGGATCTGAAGCAAGGCGATCTCTTAATTACCAAGTGATGTTCCTGGAGTAAGGCTCTGTTTGTTTCCTTTCCTGT
AAAATGCTGCATGCAAAAGTATAACACAGTAAGTAAAGAAGTCAGTTAGCCTGCACATACTAAGACCTAACCAAAGGAGCTT
ATTGTTTTTCTCCAACTTCCATGATAGGTAATTAGATAGTGGAGACCTCTGCTGGCCAATATGGTAGCCACTAACCGCAGCT
GGCTCTTCCAATTAAAATTACATAAAGCCAGAAATGTAACTCCTCTGTCTCACTTGTTATATCTCCAAGGCTGGATAGCCAC
ATGTGACTGGTGGTGGCTGGATTAGCTAGTGCATATAAAACATCACTGCAGAAAGTTCAGCTGAGCAGCACTGAGTTAGATG
GCCTCTGAAGAGGATGTCCCACGGAGAGAATCCAGAACTCAGGATCTTTTTTTTTTTTTTTCTTTGCGACAGAGTCTTGCTC
TGTCACCCAGGCTGGAGTGCAGTGGCGTGATCTCGGCTCACTGCAACTTCTGCCTCCCAGGTTCAAGCAATTCTCCTGCCTC
AGCCTCCCTAGTAGCTGGGACTACAGGCCTGTGCCAACATCCCCAGCTAATTTTTGTGTCTTTTTAGTAGAGATGGGGTTTC
ACTATGTTGGCCAGGCTGGTCTCGAACTCCTGACCTCGTGATCCGCCCCCCCTTGGCCTCCCAAAGTGCTGGGATTACAGGC
GTGAGCCACCGCGCCCGGCCAGCTCAGGATCTTTTGGGATGGTCTGTTCACCTCGCAGAAGTGGCTGGCATGAGCAATAGGA
GCCCAGAGTGGAACCAGGGCCTTCTGGTTGTCAAGTGGACTTGCTGTGTGACCTCCTGTGAGTCTCTGCCCTCTCTGAGCCT
CTCACTCCTCACCTGAGATGAGGGAGTTGGC**TGAGGTGA**TGCCGATGCCCCTCACAGCTTTGACCTCCT**ATGATTCA**GGGGC
CTGTGTGTCACCTGGTGGCACACAGATGGCAGGGAGGGTGTGAGATGGCCACACATGGGTATGAGGCAGAGCCACAGCCCCC
CCCAACACGCACATGGGCTAGTCACTGGGACACACACAGAGCTCACTCACGGCCACAGAGT**GGGCGTG**GGTTTCCCTTTTTC
CTTGTTTCTGTTTCTCTTAGCCTCGGCGCCTCCCTCCCTCCCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTGGC
TCTTTTAGCCTTGGTTTGTTACAATCTCCCTTTTCAAGCCAGCCTCTGTCCCTCCTACTCAACCTGCTTTATCTCTAGGCCT
TCCTCCTTCCTCTTCCACAGTCTGGCTTCTCACATTGTCACTCTAGGTATGCACTTCCCATTGATTTCCTCTTGCTCAGTGG
CCTCTCATCCTGCCACCCCTAGCCTTGCTGGGGACGTGAACCCTCTCCCCGCGCCTGGGAAGCCTTCTTGGCACCGGGACCC
GGAGAATCCCCACGGAAGCCAGTTCCAAAAGGGATGAAAAGGGGGCGTTTCGGGCACTGGGAGAAGCCTGTATTCCAGGGCC
CCTCCCAGAGCAGGAATCTGGGACCCAGGAGTGCCAGCCTCACCCACGGTACAATTAGACAGAGGGAAAGGGAGGGAGCCCG
GGGGTGGGGGTAGGGGAGGGTGGAGAAGCTTCCGGACTCCTTGGGGTTAAAAGGGAAGGTCCCGCCAGGGGTCCCTGGGCAG
AGGAT

-1

+1

ACCAGCGGCAGACCACAGGCAGGGCAGAGGCACGTCTGGGTCCCCTCCCTCCTTCCTATCGGCGACTCCCAGGTGAAGC
TACCTGCACCCCACCCGGGTTGGGGTGGATTGCGAGAGGATGGGTGGGGACCCCCGGGCCACAGGCAGGAGCCGGCTTA
GAGCCTCGGTTTCTCCACCTGCGGGACGCGGAAGTCCCCCCGCTGTGAGGTTGAGAAAGAGGCTCCCACTGGCTCCGAG
CCTCGGATCC

+247

TGAGGTGA: CRE ATGATTCA: AP-1 GGGCGTG: SP1

.

Fig. 12B. Nucleotide sequence of the promoter region of NES1 gene. The CAP site (+1), the AP1-binding site (ATGATTCA), the SP1-binding site (GGGCGTG) and the cyclic AMP responsive element (CRE)(TGAGGTGA) are shown in bold.

Method for Cloning and sequence analysis of the promoter region of NES1 gene. NES1 cDNA was used as a probe for the isolation of a 140-kb human genomic DNA from a bacterial

artificial chromosome (Bac) library (Research Genetics, Huntsville, AL). Bac DNA was isolated by QIAGEN Bac DNA protocol (QIAGEN, Valencia, CA), digested with various restriction enzymes and hybridized with human NES1 cDNA. These analyses allowed the isolation of a 6.5 kb Hind III fragment that included the first exon and further 5' sequences of NES1 gene. The plasmid containing this fragment was purified, sequenced and cloned into pBluescript (SK) vector (Stratagene, CA). A 354 bp Hind III-Pst I fragment corresponding to nucleotides -62 to +292 of the NES1 transcript was used as a probe to identify a single fragment of 3.1 kb. This the NES1 promoter containing fragment corresponded to sequences –2875 to +247 with respect to transcription start site and included the 73 bp exon 1 as well as part of first intron.

Demonstration of NES1 promoter activity. The pGL3 basic Luciferase expression plasmids were used to test the activity of the NES1 promoter. The NES1 promoter fragments from -2875 to -1 (del1), -1357 to -1 (del2), -1345 to -1 (del3), -800 to -1 (del4) and -292 to -1 (del5) were amplified by PCR. The other two mutants, del6 and del7 were generated by digesting the promoter with Pst I (-1457) and Sac I (-650), respectively. The fragment with appropriate size was purified from the agarose gel and subcloned into pGL3 basic promoterless plasmid as Kpn I and Bgl II fragment to generate this construct. The -2875 to +247 was also cloned and used as full length. This construct was sequenced to confirm the expected structure. To functionally localize the promoter within the 5'-untranslated sequences of the NES1 gene fragment identified above, we established a panel of luciferase reporter constructs in which various parts of the putative promoter region (Fig. 12 C) were cloned upstream of the firefly luciferase gene in promoterless pGL3 basic plasmid. The sequences of the cloned fragments were confirmed and these plasmids were analyzed by transfection in a NES1 expressing immortal mammary epithelial cell line 76NTERT (Fig. 13). CMV-βgal, encoding β-galactosidase, under a constitutive CMV promoter was used as an internal control of transfection efficiency. Forty-eight hours after transfection, the cell lysates were prepared and assayed for β-galactosidase activity by a chemiluminescence assay and the luciferase reporter activity was determined using luminometry. The luciferase activity of transfected cells was normalized relative to β-Gal activity to control for any differences in transfection efficiency. All results are presented as fold increase in luciferase activity over that observed with promoterless pGL3 basic vector. As shown in Fig. 13, the initial BamH I fragment, which included exon 1 and part of first intron exhibited relatively poor (less than 10-fold) promoter activity. Removal of exon 1 and intron 1 sequences (-2875 to -1) resulted in a marked increase in activity (about 40-50 fold). Progressive 5' truncations revealed that a fragment incorporating sequences -1354 to -1 was essentially as potent as -2875 to -1. Further truncations led to progressive loss of reporter activity with substantial (about 20 fold) activity even in the shortest fragment (-282 to -1). Similar to poor activity of the -2875 to +247 bp fragment, deletion constructs including nucleotide -1354 or -623 bp but in addition containing the exon 1 and part of intron 1 were relatively inactive. These results indicate that -1366 to -1 contain elements that are sufficient for full activity of the NES1 promoter when linked to a heterologous reporter.

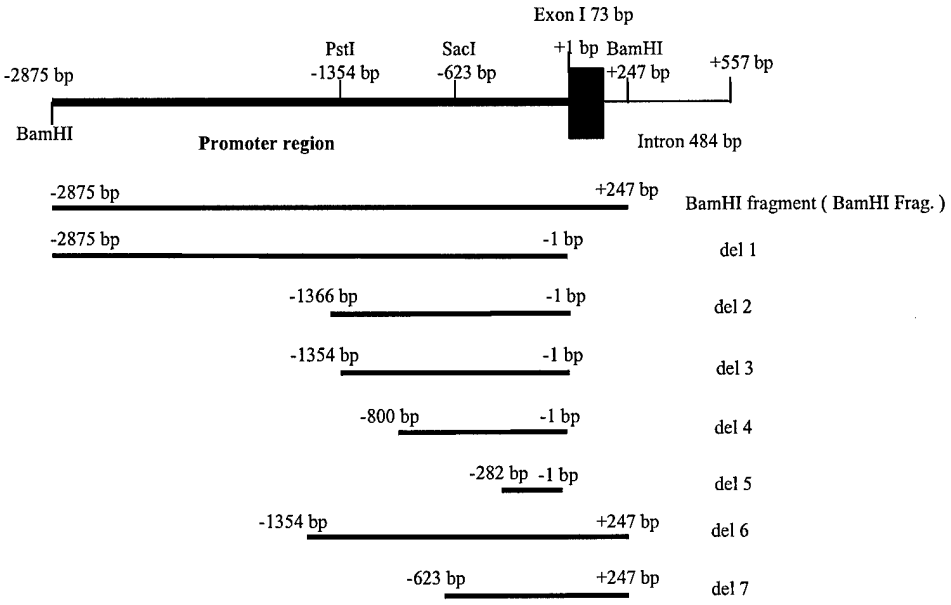


Fig. 12C. Schematic representation of the NES1 Promoter region and the various fragments. Del, deletion., bp, base pair. All mutants were generated as described below.

Method: Generation of NES1 promoter deletion luciferase reporter constructs. The 3.1 kb BamH I fragment including bases –2875 to +247 with respect to transcription initiation was cloned upstream of the luciferase gene in pGL3 basic luciferase expression plasmid. This construct is referred to as the BamH I fragment promoter. del6 and del7 mutants were generated by digesting the BamH I fragment promoter with Pst I (-1457) and Sac I (-650), respectively. The remaining NES1 promoter deletions were generated by PCR using the following primers: The bold nucleotides are for cloning these fragments into pGL3 basic.

del1 (from –2857 to –1)
sense 5' **GGTACC**GGATC CAGGAGACGATGAAGAACAATT 3'
antisense 5' **AGATCT**ATCCTCTGCCCCAGGGACCCCTGGCGGG 3'
del2 (from -1500 to –1):
sense 5' **GGTACC**TATAAAACATCACTGCAGAAAGTTCAG 3'
antisense 5'**AGATCT**ATCCTCTGCCCCAGGGACCCCTGGCGGG 3'
del3 (from -1465 to –1)
sense 5' **GGTACC**CTGCAGAAAGTTCAGCTGAGCAGCACT 3'
antisense 5' **AGATCT**ATCCTCTGCCCCAGGGACCCCTGGCGGG 3')

del4 (from -800 to –1)

sense 5' **GGTACC**GTTGGCTGAGGTGATGCCGATGCCCCT 3'

antisense 5' **AGATCT**ATCCTCTGCCCCAGGGACCCCTGGCGGG 3'

del5 (from–292 to –1)

sense 5' **GGTACC**CGCCTGGGAAGCCTTCTTGGCACCGGG3'

antisense 5'**AGATCT**ATCCTCTGCCCCAGGGACCCCTGGCGGG 3'

The primers used for mutants del 1 to del 5 included Kpn I (5') and Bgl II (3') restriction enzyme sites for cloning. PCR was performed as per protocol (denaturation I : 3 min at 94°C; denaturation II : 45 sec at 94°C, annealing : 2 min at 68°C; extension : 1 min at 72°C for 35 cycles; final extension : 10 min at 72°C). All constructs were sequenced to confirm the expected structure.

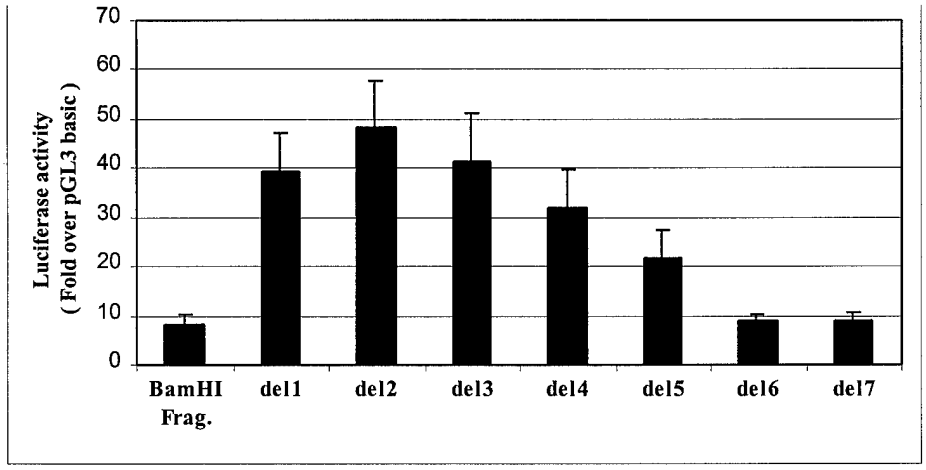


Fig. 13. NES1 promoter-luciferase activity in 76NTERT cells transfected with different fragments of NES1 promoter. X axis shows different NES1 fragments and Y axis shows luciferase activity over basic pGL3 promoter. Efficiency of transfection was controlled by β-gal assay.

Method. DNA Transfection and Luciferase Assay. Plasmids were prepared by alkaline lysis and column-purified (Qiagen Maxi Plasmid kit). About $5x10^5$ 76NTERT cells were plated per 100-mm diameter dish for 48 hours and co-transfected with 4 μg of NES1 promoter/luciferase reporter plasmid together with 1 μg of CMV-βgal (Clontech, Palo Alto, CA) (as an internal control for transfection efficiency) using the FUGENE reagent (Roche, Indianapolis, IN), according to the supplier's protocol . Forty-eight hours after transfection, the cells were washed with PBS and harvested by scraping directly into 1ml of lysis buffer (Promega, Madison, WI). The β-galactosidase activity was determined using a chemiluminescence assay according to the supplier's protocol (Clontech, Palo Alto, CA). The luciferase activity in 20 μl aliquots of cell lysates was measured by luminometry using commercially obtained reagents (Promega) and luminometer.

Analysis of NES1 promoter-luciferase activity in NES1-expressing and NES1-negative cells (New task). To assess if lack of NES1 expression in tumor cell lines may be a result of the lack of appropriate transcription factors, we examined the activity of the NES1 (-1366 to -1)-luciferase reporter in a group of NES1-expressing (76NTERT, 70E6, 21PT, Caski) and NES1-non-expressing (MDA-MB-231, MDA-MB-435, MDA-MB-453 , MCF-7, Hela and Siha) cell lines. In these experiments, cells were co-transfected with NES1 promoter (-1366 to -1)-luciferase together with PRL-SV40 reporter plasmid, which encodes a renilla luciferase protein and served as internal control for transfection efficiency. The firefly and renilla luciferase activities were assayed using the Dual-Luciferase Reporter Assay System. As expected, high NES1 promoter activity was seen in NES1-expressing 70E6 and 76NTERT cells. Notably, relatively low activity was observed in two NES1-non expressing (MCF-7 and MDA-MB-453) and a NES1-expressing (Caski) cell lines (Fig. 14). Notably, however, significant reporter activity was observed in three tumor cell lines (MDA-MB-231, MDA-MB-435 and Hela) which lacked NES1 mRNA expression. More importantly, the promoter activity in Siha cells which completely lack NES1 expression, was comparable to that in normal MECs (Fig. 14). Furthermore, the levels of promoter activity in 21PT cells, which express significant levels of NES1 mRNA, showed substantially lower activity than that in a NES1-nonexpressing (Siha) cell line. These results indicated that a defect in transacting factors was unlikely to account for lack of NES1 mRNA expression in a majority of tumor cell lines.

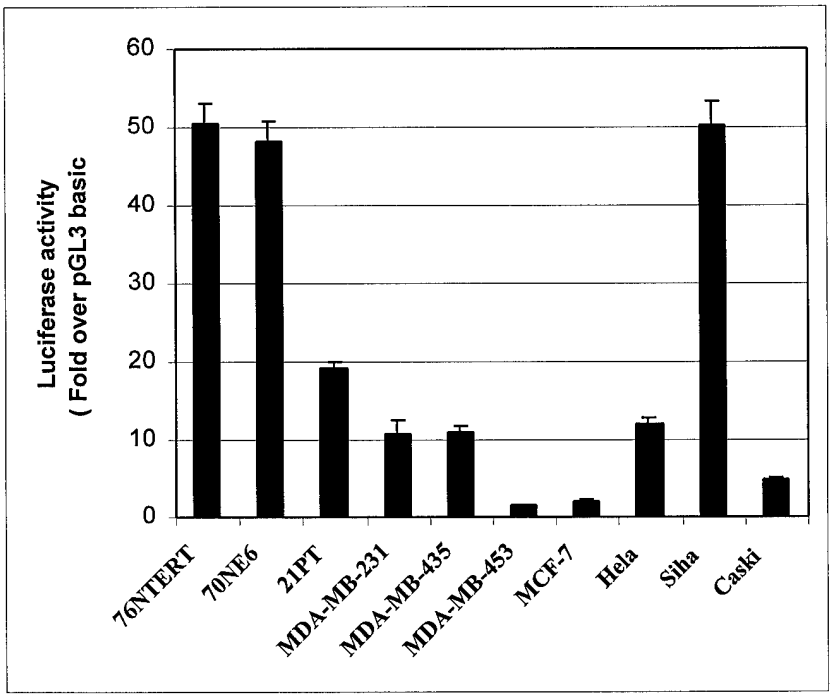


Fig. 14. Analysis of NES1 promoter-luciferase activity in NES1-expressing and NES1-negative cells. To assess the relative activity of the minimal promoter (del 2), in various cell lines, cells were transfected using either FUGENE (76NTERT, 70E6, 21PT, MDA-MB-435, MDA-MB-453, MCF-7, Hela, Siha and Caski) or Trans IT-LT1 (Mirus, MDA-MB-231) as specified by manufacturer. In these experiments, the cells received 4.9 μg of NES1 promoter(del2) /luciferase or the pGL3-basic vector (as a negative control), with 0.1 μg of renilla luciferase reporter PRL-SV40 (control for transfection efficiency). Cell lysates were prepared 48 h post-transfection and firefly and renilla luciferase activities were quantified according to the manufacturer's instructions (Promega, Dual-Luciferase Reporter Assay System). Experiments were done in triplicates, and results of at least two independent experiments are shown. Luciferase activity was normalized for efficiency of transfection (the ratio of firefly luciferase activity to renilla luciferase or to galactosidase activity). For each transfected cell line, the results were expressed as a fold ratio of those observed with the relative luminescence units over pGL3-basic vector. The mean and standard deviation (error bars) are shown.

Analysis of NES1 promoter , Exon and Intron sequences for CpG islands (New task). In view of the above results, and our previous findings that NES1-nonexpressing breast cancer cell lines do not have any mutations or deletions in the NES1 gene, we hypothesized that epigenetic alterations may underlie the lack of NES1 gene expression in tumor cells. Recent analyses have

established methylation of CpG islands in the promoter region and/or the coding regions as a frequent cause of the lack of expression of certain tumor suppressor genes, such as p16, 14-3-3σ, and RAR-β2. In vertebrate DNA, the CpG dinucleotide are under-represented occuring at only 0.2-0.25 of the expected frequency based on base composition. In contrast, gene regulatory regions are less CpG rich. Therefore, we analyzed the NES1 promoter, introns and exons sequences for the frequency of CpG islands. CpG-rich regions were defined as stretches of DNA which had both a greater than 50 % G+C content and an observed over expected frequency of CpG dinucleotides of greater than 0.6. This analyses showed that NES1 promoter was CpG-poor. Similarly, intron, and exon 1 and 6 also were CpG-poor. In contrast, exons 2 to 4 were CpG-rich, showing more than 60% G+C content an observed over expected CpG frequency more than 0.6 (Table 1). Given the particularly CpG-rich structure of exon 3 showing a 69.61 % G+C content and observed over expected CpG frequency of 1.05 (Table 1), we focussed on the analysis of methylation of this exon as a potential cause of loss of NES1 expression in tumor cells.

Table.1. Analysis of NES1 promoter, Exon and Intron sequences for CpG island

Sequence analysed	%(G+C)	O/E CpG
Promoter		
-1 to -283	63.25	0.37
-284 to -527	52.26	0.08
-528 to -771	58.85	0.24
-772 to -1015	58.85	0.29
-1016 to -1259	55.56	0.38
Exon		
I	68.49	0.47
II	68.04	0.73
III	**69.61**	**1.05**
IV	67.27	0.75
V	55.97	0.19
VI	46.77	0.27
Intron		
I	67.14	0.6
II	55.79	0.7
III	54.87	0.36
IV	62.53	0.07
V	53.66	0.12

CpG analysis of NES1 promoter and each Exon and Intron sequence. The ratio observed / expected (Obs/Exp or O/E) CpG was calculated as follows:

$$\text{Obs/Exp CpG} = \frac{\text{Number of CpG}}{\text{Number of C} \times \text{number of G}} \times N$$

Where N is the total number of nucleotides in the sequence being analyzed. The value for Obs/Exp CpG was calculated for NES1 promoter and each NES1 exon and intron sequence. For this analysis, CpG-rich regions were defined as stretches of DNA where both the value of %G+C was greater than 50, and the value of Obs/Exp CpG was greater than 0.6.

Tumor-specific hypermethylation of NES1 exon 3 (New task). To examine if lack of NES1 expression in tumor cell lines is associated with hypermethylation of the CpG island in exon 3 of the NES1 gene, we used the methylation specific PCR (MSP), a method used in previous studies to demonstrate hypermethylation of RARβ and 14-3-3σ in breast cancer cells. This method relies on selective conversion of unmethylated, but not methylated, cytosines to uracil when DNA is treated with sodium bisulfite. Uracil is subsequently recognized as thymidine by the Taq polymerase. Two pairs of PCR primers were designed to selectively yield PCR products depending on whether the target DNA was methylated or unmethylated. Amplification with the two sets of primers was carried out on sodium-bisulfite-treated DNA from a number of NES1 non-expressing and NES1-expressing cell lines.

A PCR product was only observed with primers designed to amplify unmethylated DNA in all three NES1-positive cell strains 76N and 81N. In contrast, a PCR product was specifically amplified by the methylation-specific primers in three-breast cancer cell lines (MDA-MB-231, MDA-MB-453 and MDA-MB-175) and three cervical carcinoma cell lines (Hela, Siha and Caski). A PCR product was essentially undetectable with primers designed to amplify unmethylated sequences in these cell lines (Fig. 15). A PCR product was amplified both by the methylation-specific and unmethylated primers in three-breast cancer cell lines (MDA-MB-435, BT-474 and ZR-75-1) (Fig. 15). This suggest that these three cell lines contain partial methylation of exon 3.

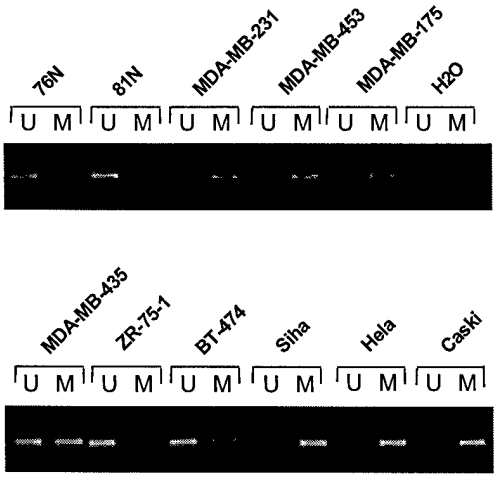


Fig. 15. Analysis of methylation in exon 3 using Methylation specific PCR (MSP). Method: One ug sodium bisulfite-treated genomic DNA was subjected to PCR amplification using the following primer combinations: The methylation-specific primers included in which nucleotides corresponding to potentially methylated Cs were retained. A 5' primers that covered the CG dinucleotides 7, 8, and 9 (5'-TTCGAAGTTTATGGCGTTTC-3') and a 3' primer that covered CG dinucleotides 21, 22 and 23 of the NES1 exon 3 (5'-TTATTTCCGCAATACGCGAC). The primer combination to amplify unmethylated DNA included in which the nucleotides corresponding to C nucleotides were changed to T (sense primer) or A (anti-sense primer), a 5' primer that correspond to CG dinucleotides 1,2 and 3 (5'-TTGTAGAGGTGGTGTTGTTT-3') and a 3' primer that covered CG dinucleotides 16,17, 18 and 19 (5'-CACACAATAAAACAAAAAACCA-3') for specific unmethylated DNA. The methylation-specific and non-methylated DNA-specific primers yielded 137-bp and 128-bp PCR products, respectively. The PCR conditions were as follows: 1 cycle of 95 °C for 5 min; 35 cycles of 95 °C for 30 Sec, 55 °C for 30 Sec and 72 °C for 45 Sec; and 1 cycle of 72 °C for 4 min.

To further examine the pattern of CpG methylation in NES1 exon 3, PCR products were amplified from sodium bisulfite-treated DNA using primers within introns flanking exon 3. The PCR products were cloned and five individual colonies were sequenced to identify methylated cytosine residues (which were not converted to uracil). All three NES1-expressing cell strains (76N, 81N and 55VN) showed lack of methylation on the vast majority of exon 3 CpG islands, with only partial methylation at CpG dinucleotides 1, 5 and 14 (and on 11 in 81N). In contrast, many CpG dinucleotides were partially or fully methylated in all ten NES1 nonexpressing cell lines (Fig. 16). Notably, the distal CpG dinucleotides (21-23) were fully unmethylated in NES1-expressing cells whereas these were invariably methylated in NES1-nonexpressing cells. These results suggest exon 3 as an important site for methylation in NES1 gene.

	NES1 Exon 3 CpG island																							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
76N	◐	○	○	○	◐	○	○	○	○	○	◐	○	○	◐	○	○	○	○	○	○	○	○	○
81N	◐	○	○	○	◐	○	○	○	○	○	○	○	○	◐	○	○	○	○	○	○	○	○	○
55VN	◐	○	○	○	◐	○	○	○	○	○	○	○	○	◐	○	○	○	○	○	○	○	○	○
MDA-MB-231	●	●	◐	◐	◐	◐	◐	◐	◐	◐	◐	◐	◐	◐	●	◐	◐	●	●	●	●	●	◐
BT-474	◐	○	◐	○	○	○	○	○	◐	◐	◐	◐	◐	◐	◐	○	◐	○	◐	◐	◐	◐	◐
MDA-MB-175	◐	○	○	○	○	○	○	○	○	○	○	○	○	◐	○	○	○	◐	◐	◐	◐	◐	◐
MDA-MB-415	◐	○	○	○	◐	◐	○	◐	○	○	○	○	○	○	○	○	○	○	◐	◐	◐	◐	◐
MDA-MB-453	◐	◐	◐	◐	◐	◐	◐	◐	◐	◐	◐	◐	◐	◐	◐	◐	◐	◐	◐	◐	◐	◐	◐
MCF-7	○	○	○	○	◐	○	○	○	◐	◐	◐	◐	◐	◐	◐	◐	◐	◐	◐	◐	○	◐	◐
MDA-MB-435R	○	○	○	○	◐	○	○	○	◐	◐	○	○	○	◐	◐	○	○	◐	◐	◐	○	◐	◐
Hela	◐	○	○	○	○	○	○	○	◐	◐	◐	◐	◐	●	◐	◐	◐	◐	◐	◐	◐	◐	◐
Siha	◐	◐	◐	◐	◐	◐	○	○	●	●	●	●	●	◐	●	◐	●	●	●	●	●	●	●
Caski	◐	○	○	○	○	○	○	○	◐	◐	◐	◐	◐	●	◐	○	◐	◐	◐	◐	◐	◐	◐

Fig. 16. Nucleotide sequencing of NES1-DNA following NaHSO3-treatment of HMECs , breast cancer cell lines and cervical carcinoma cell lines. ○, Unmethylated; ●, completely methylated; ◐, partially methylated.

Method: Analysis of DNA methylation by sequencing of sodium bisulfite-treated DNA. Genomic DNA was isolated and subjected to sodium bisulfite treatment to modify unmethylated cytosine to uracil. Bisulfite-treated DNA was subjected to PCR using the following primers: 5'-GAATGTAGTTTAGTGTTATAGTTTAG-3' (sense primer with start at NES1 nucleotide +2366) and 5'-CACACCTCCAACTATAAAAATTCC (anti-sense primer with start at NES1 nucleotide +2713). The 373-bp PCR product includes the NES1 exon 3 sequences (nucleotides +2455 to +2635). The conditions for the PCR were as follows: 1 cycle of 94 °C for 2 min; 35 cycles of 94 °C for 45 sec, 60°C for 60 sec and 72 °C for 90 sec; and 1 cycle of 72 °C for 10 min. The product was purified using a QIAGEN DNA extraction kit and ligated to a TA cloning vector (Invitrogen). Five positive clones were sequenced for each cell line using T7 and BGH primers and an Applied Biosystems automated fluorescent sequencer, according to the manufacturer's instructions.

Reversal of DNA methylation with 5-aza-dC induces NES1 expression in NES1 non-expressing tumor cells (New task). One test that has been used to causally implicate hypermethylation of DNA in tumor cell-specific lack of gene expression is treatment with demethylating agents. For example, 5-aza-dC treatment has been shown to induce de novo expression of p16, 14-3-3σ, RAR-β2 in tumor cells that did not express these genes as a result of hypermethylation. To assess if 5-aza-dC treatment induces de novo expression of NES1 in non-expressing cells, we treated representative NES1-expressing (76N, 81N, 70E6 and 81E6), and non-expressing cell lines (MDA-MB-231, MDA-MB-453, MDA-MB-435, BT-474, ZR-75-1, MCF-7, Siha and Caski), with different concentrations (10 μM, 50 μM and 100 μM) of 5-aza-dC for 48 hours and performed RT-PCR to detect NES1 expression. GAPDH was co-amplified as an internal control. Treatment of NES1 expressing cell lines did not alter the level of NES1 expression, as assessed by RT-PCR (Fig. 17 A). In contrast, 5-aza-dC treatment of NES1 non-expressing tumor cell lines resulted in dose-dependent induction (MDA-MB-231, MDA-MB-435, Siha) or increase (Caski and MCF-7) in NES1 expression as assessed by RT-PCR products. In each case, the control GAPDH message were equivalent in samples obtained from cells treated with different concentrations of 5-aza-dC treatment. In addition to cells in Fig. 17 A, we also observed similar results in two NES1-expressing (76N, 81E6) and NES1-nonexpressing (MDA-MB-453, BT-474, ZR-75-1) cells (data not shown). To further correlate the induction/enhancement of NES1 expression with 5-aza-dC-induced change in DNA methylation, we carried out PCR analysis of exon 3 methylation using primers specific for unmethylated and methylated DNA in MDA-MB-231 cells (Fig. 17 B). While untreated MDA-MB-231 cells DNA yielded a PCR product only with methylation-specific primers, treatment with increasing concentrations of 5-aza-dC led to the appearance of increasing levels of the PCR product corresponding to unmethylated exon 3 (Fig. 17 B). These results strongly implicate methylation of exon 3 in loss of NES1 expression in breast and cervical cancer cells.

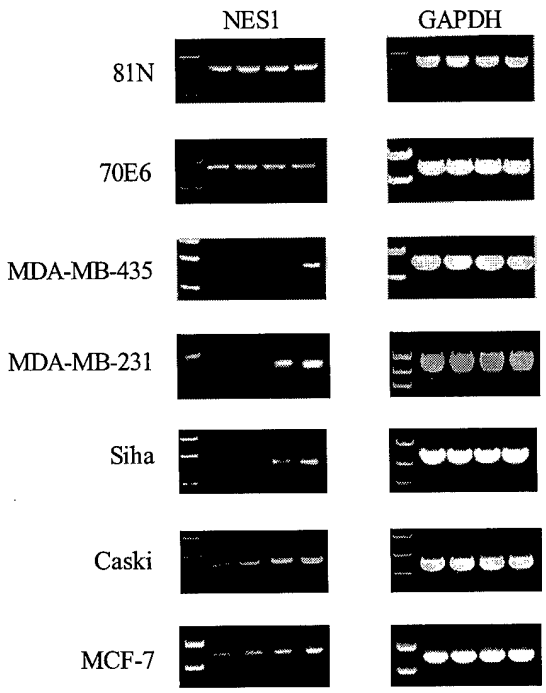


Fig. 17A. Treatment of NES1-expressing and NES1-negative cells with 5-aza-dC. Cells were seeded at a density of 5×10^5 /100-mm dishes, cultured for 48 hours and treated with 10, 50 or 100 μM 5-aza-dC (Sigma) or left untreated. Forty-eight hours after treatment, cells were washed with phosphate buffered saline (PBS), fresh medium was added and cells were further incubated for 2 days before isolating total cellular RNA and genomic DNA.

RT-PCR: RNA was treated with RNase-free DNase I (GIBCO-BRL or Boehringer-Mannheim) (0.5-1 μg/μl) for 1 h at 37°C, followed by RNeasy Mini Protocol for RNA Cleanup (QIAGEN). Reverse transcriptase (RT) reactions were performed in 2 μg DNase-treated RNA, 1μl Oligo $(dT)_{12\text{-}18}$ using SUPERSCRIPT™ II RNase H⁻ Reverse Transcriptase Kit protocol (GIBCO-BRL). PCR was then performed using the NES1-specific primers 5'-CTCGAGTAGGGGATGATCACCT-3' and 5'-GCTTCAGTACAGGCAGAGAA-3' and the Advantage cDNA PCR kit (Clontech). The PCR conditions were as follows: 1 cycle of 94°C for 2 min; 35 cycles of 94°C for 45 sec, 62°C for 1 min and 72°C for 1min 30 sec. The GAPDH gene and a "housekeeping" ribosomal protein gene 36B4 were co-amplified as internal controls; primers for GAPDH were 5'-TGAAGGTCGGAGTCAACGGATTTGGT-3'(sense) and 5'-CATGTGGGCCATGAGGTCCACCAC-3'(antisense), or for 36B4 were 5'-GATTGGCTACCCAACTGTTGCA-3'(sense) and 5'-CAGGGGCAGCAGCCACAAAGGC-3' (antisense). The PCR products were resolved by electrophoresis on 1% agarose gels.

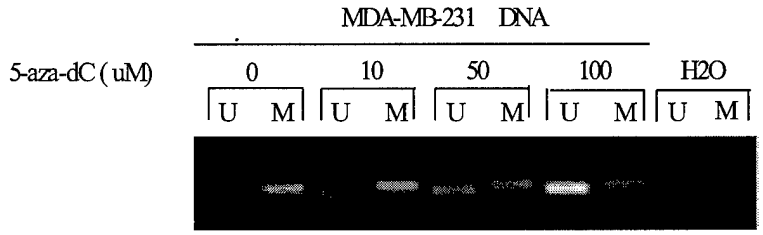


Fig. 17 B. MSP analysis of Exon 3 in DNA from MDA-MB-231 breast tumor cells before and after treatment with 5-aza-dC. Treatment of cells with 5-aza-dC and MSP were performed as above.

Analysis of NES1 expression and methylation in primary breast tumor specimens (New Aim). To assess if the inactivation of NES1 expression through hypermethylation is indeed a cancer related epigenetic phenomenon rather than an in vitro culture-induced alteration, we examined NES1 expression and exon 3 methylation in ten primary breast tumor samples. Total RNA was isolated from tumor tissues and analyzed for NES1 expression by RT-PCR. As expected, NES1-expressing normal MEC strains 184 and 437, and a benzopyrene-immortalized mammary epithelial cell line 184A1 (used as positive controls) showed easily detectable NES1 PCR product, whereas MDA-MB-231 (used as a negative control) showed no product (Fig. 18A). Notably, two freshly isolated organoids derived from normal mammoplasty specimens showed easily detectable NES1 PCR product (Fig. 18 A). In contrast, 4 out of 11 primary breast carcinomas showed no NES1 PCR product, whereas a product was detectable in the remaining seven samples.

The NES1-expressing and non-expressing primary tumor samples were used in further analyses using methylation-specific PCR primers to explore if the lack of NES1 expression in these tumor specimens correlates with methylation of exon 3. These results showed that tumor samples with detectable NES1 RT-PCR product (3, 11, 13, 30, 42 and 63) did not yield a PCR product with exon 3 methylation-specific primers whereas a product was observed with primers specific for unmethylated product. In contrast, samples with no detectable NES1 RT-PCR product (9, 15, 21 and 36) showed PCR products corresponding to both methylated and unmethylated NES1 exon 3 (Fig. 18 B). One specimen (# 7) showed NES1 expression by RT-PCR (Fig. 18 A), and yielded PCR products corresponding to both unmethylated and methylated NES1 exon 3 (Fig. 18 B). It is likely that the presence of an RT-PCR product in this sample reflects the high sensitivity of the RT-PCR method which may allow detection of extremely low levels of NES1 expression.

Sodium bisulfite treated DNA from the 8 tumor samples described above was also subjected to PCR amplification of the exon 3 to identify the pattern of CpG methylation. As seen in Fig. 18 C, all of the tumor samples with no expression of NES1 (9, 15, and 36) showed higher levels of methylation, particularly in the distal CpG islands. In contrast, those samples with detectable NES1, there was no methylation of exon 3 (3, 11, 30, and 42) (Fig. 18 C).

Interestingly, #7 specimen that expressed NES1 and showed both methylated and un methylated PCR product, showed complete methylation of CpG islands 1, 14, and 21(Fig. 18 A-C).

In addition to tumor specimens described above, we also carried out methylation-specific PCR analysis of DNA isolated from four other specimens where samples of tumor as well as adjacent pathologically normal tissue were available. These analysis showed methylation of exon 3 in 3 out of 4 tumor samples but only unmethylated NES1 was seen in the normal tissues (Fig. 18 D). Taken together, these data support the conclusion derived from analyses of in vitro cultured cell lines that DNA methylation provides a mechanism for tumor-specific loss of expression of NES1 gene.

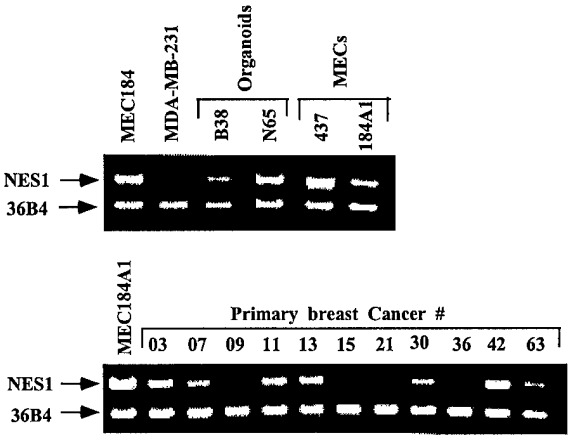


Fig. 18 A. RT-PCR analysis of NES1 expression in primary breast cancers.
184 and 437 (two normal MECs) and 184A1 (immortal cell line) were used as positive control, and MDA-MB-231 cell line was used as a negative control. B38 and N65 are normal tissue organoids. 36B4 is used as a internal control. Total cellular RNA from breast tissues was extracted from breast tissues using Triazol. RT-PCR was carried out as above.

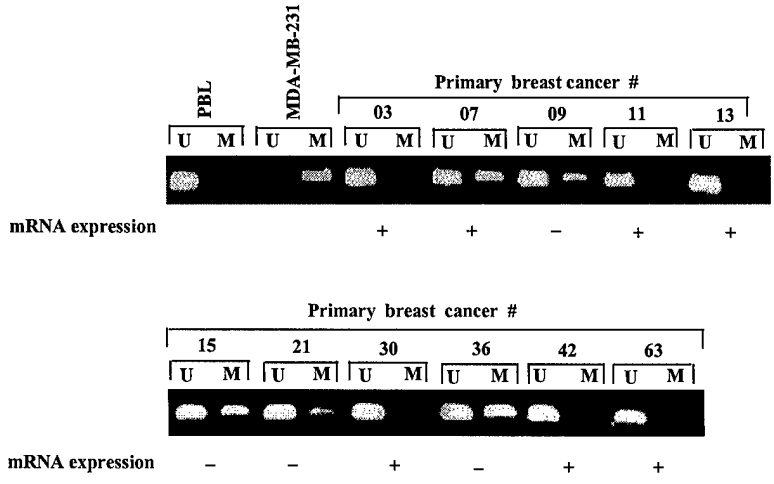


Fig. 18 B MSP analysis of DNA from primary breast cancers. Microdissected tumor tissues from 11 primary breast cancer specimens were used to isolate DNA. MSP was carried out as described above.

	1 2 3	4 5 6	7	8	9 10 11 12 13 14	15 16 17 18 19	20 21 22 23
03	○○○	○ ○○	○	○	○○○○○ ◐	○ ○ ○ ○○	○○○ ○
07	●○○	○ ○○	○	○	○○○○○ ●	○ ○ ○ ○○	○●○ ○
09	◐◐◐	○ ◐○	○	○	○○○○○ ◐	◐ ◐ ◐ ◐○	◐◐◐ ◐
11	○○○	◐ ○○	○	○	○○○○○ ○	○ ○ ○ ○○	○○○ ○
15	●●●	◐ ◐○	◐	○	◐◐◐◐◐ ◐	◐ ● ◐ ◐◐	◐◐◐ ◐
30	○○○	○ ◐○	○	○	○○○○○ ○	○ ○ ○ ○○	○○○ ○
36	◐○○	○ ○○	○	○	◐●●●◐ ●	◐ ◐ ◐ ◐●	◐●● ●
42	◐○○	○ ○○	○	○	○○○○○ ◐	○ ○ ○ ○○	○○○ ○

Fig. 18 C. Exon 3 sequencing following NaHSO3-treatment of eight primary breast cancer tissues. ○, Unmethylated; ●, completely methylated; ◐, partially methylated.

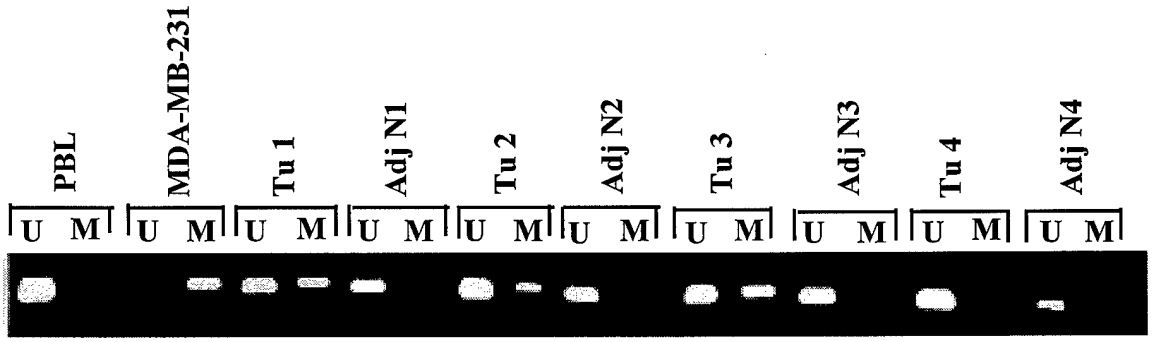


Fig. 18 D. MSP of Exon 3 in 4 primary breast cancers and adjacent normal breast tissue. Tu: Primary breast cancer tissue, Adj N: Adjacent tissue histopathologically normal. MSP was carried out as described above.

Summary of the unpublished work presented above

NES1 (Normal Epithelial Cell Specific-1) gene is expressed in normal mammary epithelial (MECs) and prostate cells, but its mRNA and protein expression is dramatically decreased in most established breast and prostate cancer cell lines, even though no major deletion or rearrangements in NES1 gene are found. Here, we have cloned and characterized the active promoter region of NES1. Using a luciferase reporter system, we demonstrate that most tumor cell lines are able to support full or partial transcription from NES1 promoter, suggesting a role of cis-acting mechanisms of loss of NES1 expression. We show that hypermethylation of NES1 gene represents one such mechanism. While NES1 promoter is CpG-poor, exons 2 to 4 are CpG rich, with a high density of these sequences within exon 3. Using PCR and sequence analysis of sodium bisulfite-treated genomic DNA, and PCR with methylation-specific primers, we

demonstrate a strict correlation between exon 3 hypermethylation and loss of NES1 expression in a panel of breast and cervical cancer cell lines. Importantly, using a limited set of primary breast tumor samples, we demonstrate that hypermethylation of exon 3 correlates with lack of NES1 expression. Treatment of NES1-nonexpressing cells with a demethylating agent led to reexpression of NES1, suggesting a potentially important role of hypermethylation in loss of NES1 expression. We suggest that loss of NES1 expression and hypermethylation can serve as tumor markers in breast cancer and possibly in other cancers. The assays developed here should facilitate future analyses to evaluate NES1 expression/hypermethylation as potential marker for diagnosis and/or prognosis of breast and other cancers.

Establishment of in-situ hybridization method to detect NES1 mRNA expression in breast tumor specimens (Aim I-2). As shown above, NES1 is a secreted protein therefore immunohistochemistry using anti-NES1 polyclonal antibodies did not give us confidence in analyses of NES1 expression in normal and tumor tissues. Therefore, we developed in situ hybridization using sense and antisense NES1 probe. For this purpose, a NES1 cDNA fragment containing nucleotides 1-1069 was cloned into pBluescript vector (Stratagene, La Jolla, CA). The plasmid was linearized with HindIII or XbaI to generate antisense and sense templates, respectively. The transcription reactions were carried out using Maxiscript T7/T3 kit (Ambion, Austin, TX) using Biotin-16-UTP (Boehringer Mannheim) to generate biotin-labeled probes.

Paraffin embedded breast tissue slides received from the Department of Pathology, were subjected to in-situ hybridization using mRNA*locator*-Hyb Kit (Ambion, Austin, TX). Tissues were deparaffinized by submerging in xylene and rehydrated in decreasing concentrations of alcohol. Proteinase K digestion of the slides (2 μg in 50 μl Tris Buffer, pH 7.5, per slide) was performed at 37°C for 30 min. 20 ng/slide of probe was used to perform the hybridization at 50°C for overnight. Slides were then washed once with 2x in-situ solution for 10 min. at 50°C, followed by three washes with 1x in-situ wash solution, 10 min. each at 50°C. Detection of in-situ hybridization was performed using mRNA*locator*-Biotin kit (Ambion, Austin, TX). Slides were incubated with Streptavidin-Alkaline Phosphatase (50 μl of 1:3000 dilution/slide) at 37°C for 30 min. Hybridization signal was detected using 50 μl of NBT/BCIP solution for each slide at 37°C for 1 hr. The reaction was terminated for color development by giving two washes of 4 min. each in nuclease-free water. Tissues were dehydrated in ascending concentrations of alcohol and cleared in xylene. Tissue sections were permanently mounted with Permount (Fisher Scientific, Fair Lawn, NJ).

As seen in Fig. 19, NES1 was clearly detected in normal mammary ducts with the antisense probe but not with the sense probe. In contrast, NES1 mRNA was undetectable in six out of six infiltrating ductal carcinomas analyzed so far (Fig. 19, and data not shown).

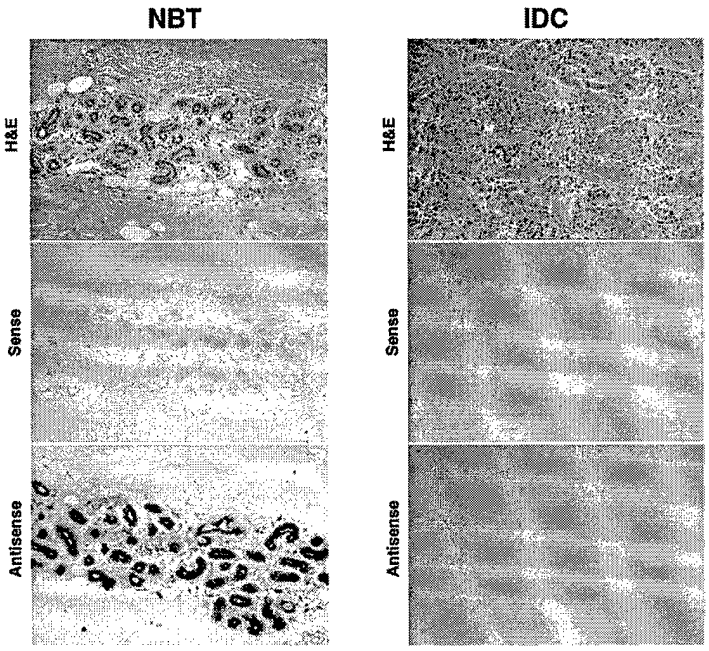


Fig. 19. NES1 mRNA expression in normal ducts but not in infiltrating ductal carcinomas. In situ hybridization was carried out as described above. H&E, hematoxylin and eosin staining. Sense and antisense probes are described above. NBT, normal breast tissue; IDC, infiltrating ductal carcinoma.

Use of wild-type and serine mutants of NES1, we demonstrate that NES1 lacks proteolytic activity (Aim III).

Construction of NES1 mutants using site directed mutagenesis (AIM III). The predicted NES1 polypeptide shows high homology with various families of serine proteases. Importantly, residues surrounding the active site serine is highly conserved (R-D-S-G-G in trypsin and S-D-**S**-G-G in NES1). The other two amino acids of the catalytic triad of serine proteases (equivalent to trypsin residues H57 and D102) are also strictly conserved; the corresponding residues in NES1 are H86 and D137. NES1 also possesses essentially all of the residues that form the specificity pocket of serine proteases, which is involved in substrate binding and specificity. Interestingly, homology search with other proteases also revealed unique amino acid regions in NES1 polypeptide at positions 44-48, 123-130 and 148-151. These homology comparisons provided a clear rationale for mutational analysis of the role of protease activity in NES1 function. Full length NES1 cDNA in pCMV-neo plasmid (used for transfection in MDA-MB-231 cells) was used to perform site-directed mutagenesis using Quick Change site-directed mutagenesis kit (Stratagene). Mutagenic oligonucleotide primers were designed individually according to the desired alanine substitution or point deletion of critical amino acids. The primers were of 30-40 bases in length having desired mutation in the middle of the primer. The melting temperature (Tm) was designed to be greater than 78°C and the primers had a minimum of 40 % GC content. The following mutants were generated.

Mutants of catalytic triad: S229A, ΔS229, D137A, ΔD137, H86A, ΔH86.

Mutants of predicted cleavage site for activation : R42A, ΔR42

Mutants of Substrate binding domain: D223A, ΔD223, P224A, ΔP224, G241A, ΔG241, L243A, ΔL243, W245A, ΔW245
Mutants in conserved residues surrounding catalytic serine: G230A, ΔG230

Expression of an epitope-tagged NES1 protein in insect cells (Aim III-1).
Using the polymerase chain reaction (PCR), we have generated cDNA expression plasmids that are predicted to append an epitope tag for anti-myc antibody (9E10) at the N- or C- terminus of NES1 protein. Tagged and untagged NES1 constructs were cloned into pBacPAK6 baculovirus vector (Clontech) and introduced into insect cells (IPLB-Sf21). Expression of 3' and 5' tagged NES1 was demonstrated using immunoblotting of the insect cell supernatants with anti-myc and anti-NES1 antibodies. As shown in Fig. 20, both 3' and 5'myc tagged NES1 were expressed and secreted into culture supernatant of infected insect cells.

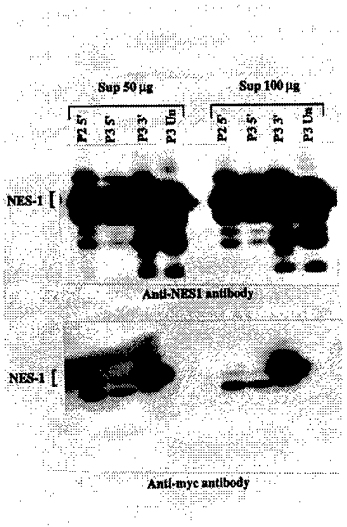


Fig. 20. Western blot analysis of NES1 protein in culture supernatants of insect cells transfected with untagged or myc epitope-tagged NES1. Untagged or myc-epitope tagged NES1 constructs were transfected into insect (Sf21) cells by calcium phosphate method. Sf21 cells were propagated as monolayer in T150 flasks in Grace's insect cell medium supplemented with yeastolate, lactalbumin hydrolysate and 10% fetal calf serum at 27°C without CO2. Upon reaching 60-70% confluency cells were infected with passage 2 or passage 3 virus expressing untagged NES1 or 5' or 3'myc-tagged NES1 protein. After 48 hours of infection, the medium was collected. 50 or 100 µg of protein from unconcentrated culture supernatants was resolved by a SDS-10 %-PAGE and transferred to a PVDF membrane. Membranes were immunoblotted with anti-NES1 antiserum (above panel) or anti-myc (lower panel) mAb (9E10) followed by goat anti-rabbit IgG or rabbit-anti-mouse conjugated to horse-radish peroxidase. Detection was by enhanced chemiluminescence. Note that immunoblotting of supernatants with anti-myc antibody (9E10) and anti-NES1 antibody shows NES1 proteins of expected size (30 kDa) with both 3' and 5' tagged constructs. Supernatants from cells transfected with untagged NES1 served as control (lower panel). Immunoblotting of supernatants with NES1 antiserum shows NES1 protein of expected size in all untagged as well as 3' and 5' tagged construct transfected cells (above panel).

Purification of NES1 protein (Aim III).

The results presented above demonstrated that we have successfully expressed NES1 as a C-and N- terminal myc-tagged protein in Sf21 insect cells (Fig. 20). This system is known to allow proteins to undergo post-translational modifications in eukaryotic cells and yields large quantities of enzymatically active proteins in numerous situations. As shown in Fig. 20, Sf21 cells expressed and secreted large quantities of NES1 protein. For purification of NES1 protein five T150 flasks of sf21 cells were infected with third progeny of BacPAK6 cells transfected with 3'-myc tagged NES1 (titre about $2X10^8$ Pfu/ml). 48 hours post-infection, the medium was

collected. To prevent metal ion catalyzed oxidation of proteins, EDTA was added to a final concentrations of 1 mM. Protein concentration, measured was 2.38 mg/ml (total protein was 587 mg). Ammonium sulphate was added to give 25 % saturation (144 g/litre). Precipitate was dissolved in a buffer containing 350 mM NaCl and 10 mM Tris, pH 7.4. Volume of the supernatant was measured and 429 g/litre ammonium sulphate was added to give a final saturation of 85%. The 85% ammonium sulfate precipitate was dissolved in 350 mM NaCl and 10 mM Tris and was dialyzed in 10,000 M.W. cutoff dialysis tubing against 10 mM Tris and 150 mM NaCl to remove excess salts. The unconcentrated supernatant (control lane 1), 25 % precipitates, 85 % precipitates and 85 % supernatant were analyzed for the presence of NES1 by Western blotting (Fig. 21). The amount of protein in 25 % ammonium sulfate precipitate was 1.92 mg (0.3 % of original amount) as compared to 49.68 mg (8.5 % of original amount) in 85 %. Therefore, for further studies 85 % ammonium sulfate precipitate was used (Fig. 21).

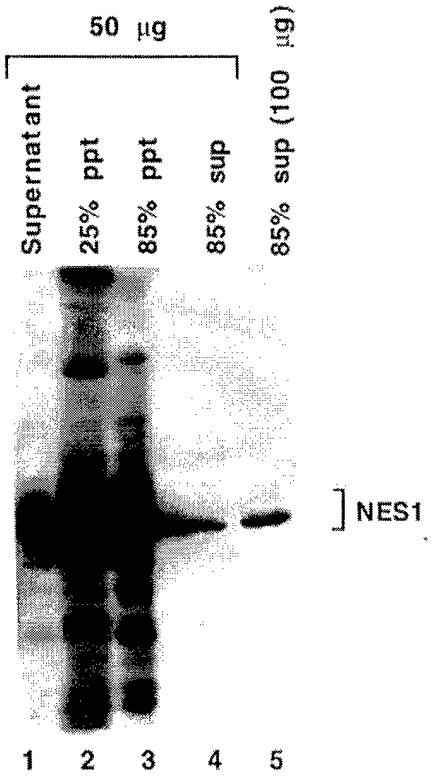


Fig. 21. Western blot analysis of NES1 protein in supernatants of insect cells transfected with 3'-myc tagged NES1. Cells were grown, their supernatant were immunoblotted with anti-NES1 antibody. Lane 1, NES1 protein in supernatants collected from virus infected Sf21 cells before ammonium sulphate fractionation; Lanes 2 and 3, NES1 in precipitate at 25 % and 85 % saturation of ammonium sulfate. Lanes 4 and 5 show residual NES1 protein in 50 µg or 100 µg protein samples of the supernatant remaining after 85 % ammonium sulfate precipitation. Lanes 1-4 were loaded with 50 µg of protein samples. Although 25 % and 85 % ammonium sulfate precipitate when loaded equally show similar quantity of NES1 protein, the total amount of protein with 25 % precipitate was 26 fold lower than in 85 % precipitate.

The 85 % ammonium sulfate precipitate of the above Sf21 culture supernatant was loaded onto a 40X2.5 cm SP-Sepharose fast flow ion exchange column at a flow rate of 3 ml/min. The column was equilibrated with 50 mM sodium phosphate buffer, pH 7.2. After washing with four volumes of buffer, the bound proteins were eluted with a linear gradient of 1 M KCl. O.D. at 280 nm was monitored on fractions using an attached UV monitor. The plot of the UV absorbance (Fig. 22) showed that the bound proteins eluted as a broad peak over 90 minutes. 6 ml fractions were collected and analyzed by immunoblotting with anti-NES1 antibody (Fig. 23). NES1 was detected in fractions 16-20 out of 25 fractions analyzed. Fractions corresponding to peak NES1 immunoreactivity (17-19) and 2 surrounding fractions (18 and 20) were concentrated using centricon 10 membrane (molecular weight cut off 10,000). 40 μg protein samples of concentrated fractions were run on 10% SDS-PAGE and protein bands were localized by silver staining (Fig. 24). Silver staining showed that the peak NES1 fractions are relatively free of contaminating protein. Second peak after NES1 peak when tested in Western blot as well as silver staining showed no detectable NES1 protein (data not shown).

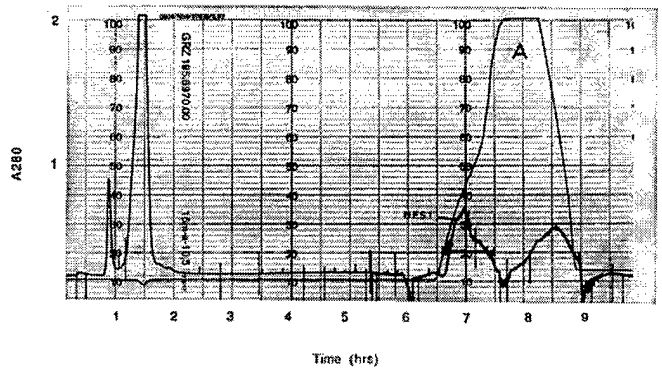


Fig. 22. Elution profile of the polypeptides bound to SP sepharose fast flow column loaded with 85 % ammonium sulfate precipitate of NES1 containing Sf21 cell supernatant. 40 X 2.5 cm column was loaded with 10 mg of 85 % ammonium sulfate precipitate of Sf21 cells infected with 3'myc-tagged NES1. The column was washed with four volumes of phosphate buffered saline (over 6 hours) and eluted with a salt gradient (0-1 M KCl in 50 mM NaPO4). 45 fractions between hour 6 to 7:30 containing the peak O.D. 280 were processed further for anti-NES1 immunoblotting (Fig. 23). Peak labelled 'A' depicts linearity of the gradient.

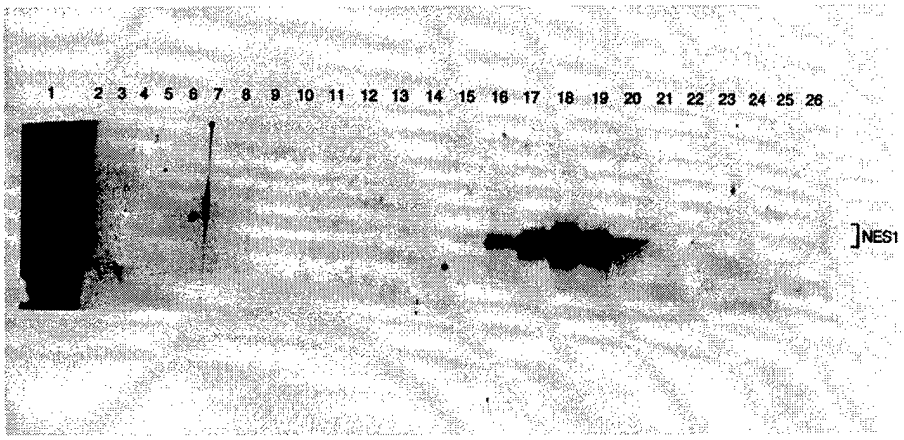


Fig. 23. Western blotting of the column fractions. 100 µl of each fraction (45 fractions, two fractions pooled into one, labelled as fraction # 4-26) was run on 10 % SDS PAGE, immunoblotted with anti-NES1 antibody and processed for Western blot as above. Fraction 1, positive control; Fractions 16-20, peak of NES1 (as indicated). Note that only ten fractions (16-20) showed NES1 protein.

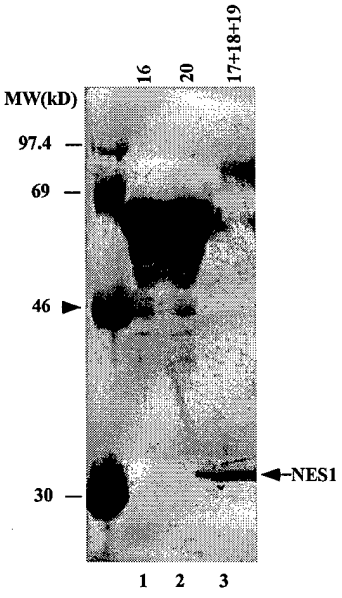


Fig. 24. Silver staining of fractions 16-20 eluted from SP-Sepharose column by KCl. As shown in Fig. 23 fractions 17,18 and 19 showed majority of NES1 protein. These fractions were pooled together, and fraction 16 and 20 were used separately. All of these fractions were concentrated 10 fold using centricon filter 10 (M.W. cutoff of 10,000) and then 40 µg protein samples of each were loaded on a 10 % SDS-PAGE followed by silver staining. Note that pooled fractions 17,18

and 19 together showed a major band of 30 kDa corresponding to the NES1 protein, whereas fraction 16 and 20 showed primarily the contaminated proteins and undetectable NES1 protein.

Further purification of NES1 protein (AIM III). Further purification of NES1 protein was achieved by subjecting NES1 protein obtained from ion exchange chromatography to Gel filtration chromatography. For this purpose, fractions containing NES1 from ion exchange column were pooled, concentrated and desalted using Centricon 10. Biogel 60 (Biorad, M.W. cutoff 60,000 kDa) was soaked in 10 mM Tris, 150 mM NaCl buffer overnight and packed in a 1.0 cm x 50 cm column at a flow rate 70 µl/min. The column was calibrated using Pharmacia's low molecular weight gel filtration standards. Ion exchange chromatography-purified and concentrated NES1 samples was loaded on Biogel column, run in the above buffer and O.D. 280 nm was monitored (Fig. 25 A). 50 µl of fractions having O.D. 280 nm were run on 10% SDS PAGE and protein was visualized by immunoblotting (Fig. 25 B). Fractions 1-9 were analyzed by silver staining (Fig. 25 C). Note that fractions 6,7 and 8 showed only one band of 30 kDa corresponding to the NES1 protein.

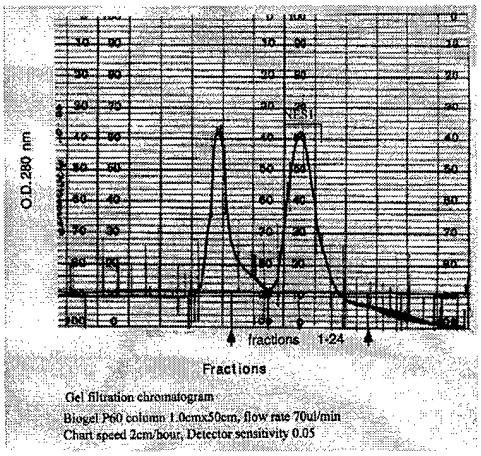
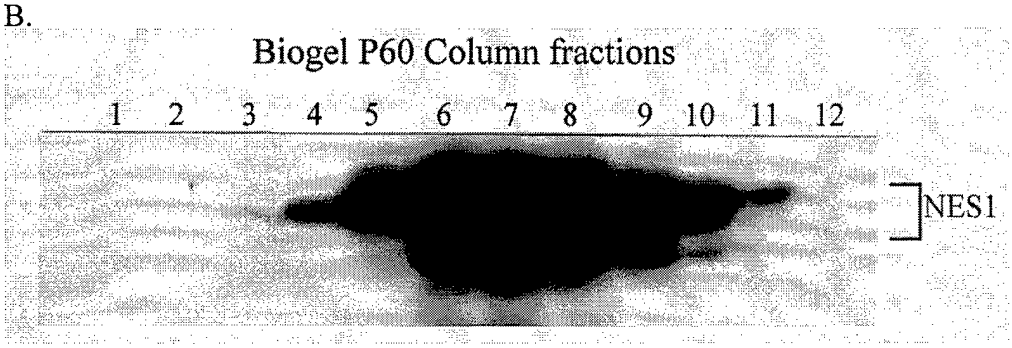


Fig. 25. (A) Gel filtration chromatography. Fractions containing NES1 from ion exchange column were pooled, concentrated and desalted using Centricon 10. Biogel 60 (Biorad, M.W. cutoff 60,000 kDa) was soaked in Tris 10 mM, NaCl 150 mM buffer overnight and packed in a 1.0 cm x 50 cm column, flow rate 70 µl/min. The column was calibrated using Pharmacia's LMW gel filtration standards. NES1 purified and concentrated from ion exchange chromatography was loaded on Biogel column, run in the same buffer and O.D. 280 nm was monitored.

B.



C.

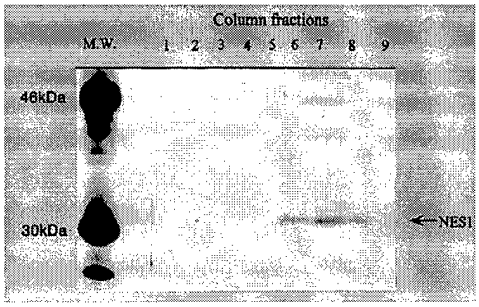


Fig. 25 . (B) 50 µl of fractions having O.D. 280 nm were run on SDS-10.5% PAGE, and transferred to PVDF membrane. Membranes were immunoblotted with an anti-NES1 antiserum followed by goat anti-rabbit IgG conjugated to horse-radish peroxidase. Detection was by enhanced chemiluminescence **(Fig. 25 C)**. 50 µl of fractions having O.D. 280 nm were run on SDS-10.5% PAGE, and protein was visualized with the help of silver staining. Note that fractions 6,7 and 8 showed only one band of 30 kDa corresponding to the NES1 protein.

Characterization of purified NES1 protein (Aim III) The overall amino acid sequence of NES1 shows a 50-63 % sequence identity with known human serine proteases. A neural network- based signal peptide and cleavage site prediction program (http://www.cbs.dtu.dk/services/signalP/) predicted that NES1 cDNA encodes a polypeptide with a putative signal peptide (amino acid 1-33), with potential signal peptide cleavage site between amino acid positions 33 and 34 (Ala-Ala). The predicted 33 amino acid signal peptide is longer than the majority of known serine proteases. A potential Trypsin- susceptible cleavage site at position 42 (Arg) was also predicted.

To determine the actual N-terminal sequence of the mature NES1 protein, we subjected the NES1 protein purified from the insect cells, as well as from the NES1 transfected MDA-MB-231 cell conditioned media, to amino terminal sequencing . In both cases, five cycles of sequencing revealed the sequence ALLPQ corresponding to NES1 sequence 34ALLPQ38 that follows the predicted signal peptide (Fig. 26) . These results directly establish that, similar to other serine proteases, the hydrophobic signal sequence of the NES1 polypeptide is cleaved at Ala-Ala signal cleavage site prior to it's secretion. Furthermore, the secreted NES1 protein retains the 8 aa pro-peptide preceding the potential cleavage site and hence is expected to be proteolytically inactive.

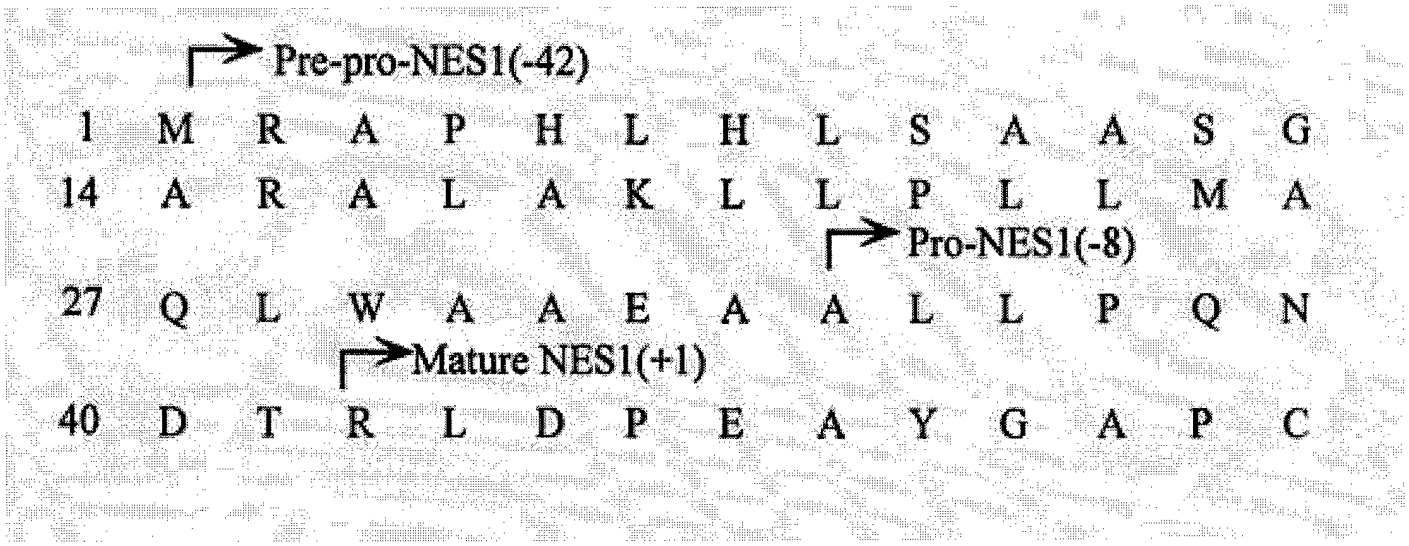


Fig. 26. Amino acid sequences of N-terminus of NES1. Pre-pro-NES1 contains a hydrophobic signal peptide (aa 1-33) that gets cleaved during secretion. Pro-NES1 contains eight amino acids (34-42) that should be cleaved to activate NES1 to function as a protease.

Examination of proteolytic activity of NES1 (Aim III). Next, we analyzed the protease activity of purified full-length NES1 and its two serine mutants (S229A and del229) using a universal serine protease substrate casein resorufin. These studies showed extremely low protease activity in both NES1 and its mutants. Based on the sequence homology of NES1 with other active serine proteases, we predicted that NES1 needs to be activated at R42 in order for it to be a potentially active protease. For this purpose, we performed limited proteolysis (to remove pro-peptide, amino acids 34-42) using low concentrations of trypsin or glandular kallikrein , which are known to have arginine restricted specificity. After each of these treatments, we tested the protein on SDS-PAGE and the protease activity was measured using a universal protease substrate, casein resorufin (Boehringer Mannheim). As expected very low concentration (100 ng) of trypsin alone (used as a control) showed high protease activity (Fig. 27 and data not shown). In contrast, 5 μg each of NES1 and its mutants showed extremely low protease activity (Fig. 27). In addition, we also carried out NES1 protease reactions at different ranges of pH (a range of 2-9) and temperature. Next, we examined if NES1 has activity against a chymotrypsin substrate Suc-Ala-Ala-Pro-Phe-pNA. As expected low doses of chymotrypsin (400 ng) showed protease activity. In contrast, 5μg each of NES1 and its mutants showed no

protease activity (Fig. 28). We also examined a number of para-nitro-anilide synthetic substrates (Diapharma Group Inc.) that are known to be specifically cleaved by plasmin (H-D- Val-Leu-Lys-pNA), trypsin (H-D-Ile-Pro-Arg-pNA), tissue plasminogen activator (H-D-Val-Phe-Lys-pNA), glandular kallikrein (H-D-Val-Leu-Arg-pNA), plasma kallikrein (H-D-Pro-Phe-Arg-pNA), elastase (<Glu-Pro-Val-pNA), urokinase (pyro-Glu-Gly-Arg-pNA) and chymotrypsin(MeO-Suc-Arg-Pro-Tyr-pNa). The untreated NES1 or treated with trypsin or glandular kallikrein displayed no detectable hydrolytic activity against any of the synthetic substrates listed above (data not shown). Each of these substrates was clearly cleaved by their respective protease (data not shown). These data suggested either NES1 has no protease activity or NES1 is not able to be cleaved of pro sequences and therefore is inactive.

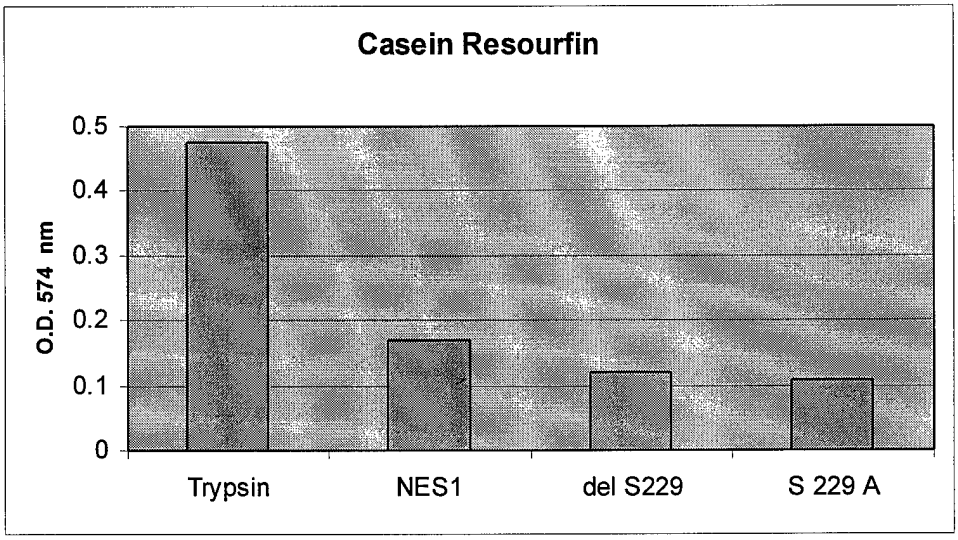


Fig. 27. Proteolysis of Casein resourfin substrate by NES1 and its serine mutants: Five μg of pro-NES1 or NES1 mutants were incubated with 0.5 μg of human glandular kallikrein (hK2), at 37^{0}C for one hour. The reactions were terminated by adding ten fold excess of aprotinin. Casein resorufin was then added to the reaction mixture and incubated further for two hours, the reaction was stopped with TCA and the absorbance was read at 574 nm. O.D., optical density. 100 ng of trypsin was used as a positive control.

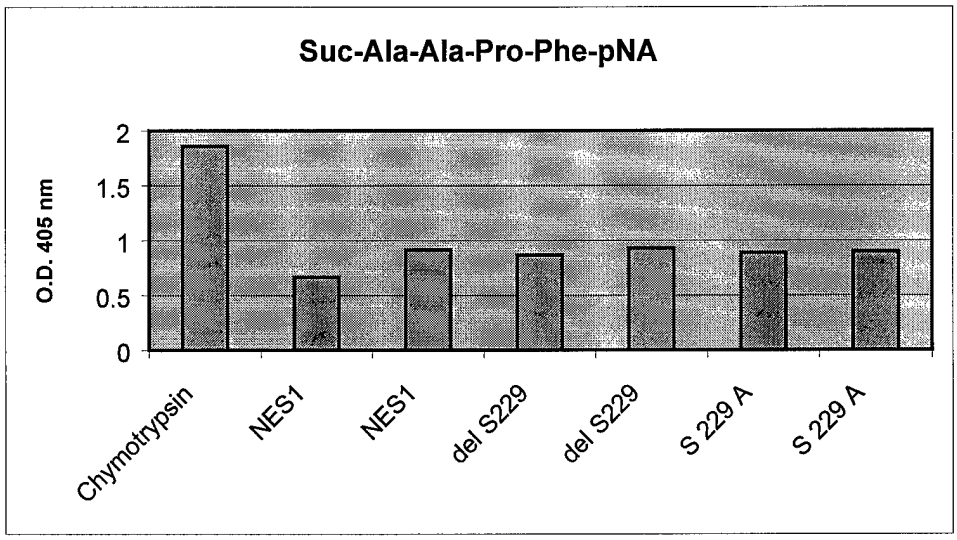


Fig. 28. Proteolysis of substrate Suc-Ala-Ala-Pro-Phe-pNA by NES1 and its serine mutants: Five micrograms of NES1 or its serine mutants were activated with hK2. The reaction was terminated by adding ten fold excess of aprotinin. The substrate was then added to the reaction mixture and the release of paranitroaniline was measured at 405 nm. 400 ng of chymotrypsin was used as a positive control.

Generation of pro-EK-NES1 and pro-EK-NES1-Flag and EK cleavage of recombinant protein (Aim III). Although above mentioned experiments clearly showed that NES1 lacks protease activity, one possibility remained that above treatment to activate Pro-NES1 may not activate NES1. As the difference of predicted activated NES1 and pro-NES1 is only 8 amino acid, we could not separate these two forms on SDS-PAGE gels for sequencing. We therefore introduced enterokinase (EK) site at R42. Using a polymerase chain reaction approach, the EK-susceptible peptide Asp-Asp-Asp-Asp-Lys was inserted into the NES1 cDNA between the domain that encodes the pro-peptide and the N-terminus of mature NES1. EK is a highly specific enzyme that recognizes Asp-Asp-Asp-Asp-Lys motif. It was anticipated that the secreted recombinant NES1 could be activated by EK treatment. In addition, to facilitate the purification of the recombinant NES1 with an anti-Flag IgG antibody, a second construct (pro-EK-NES1-Flag) was created that also contained 8-residue Flag peptide (Asp-Tyr-Lys- Asp-Asp-Asp-Asp-Lys) at its C terminus. Both cDNA constructs were inserted in the correct orientation into the multiple cloning sites of PCMV and pBacPAK vectors for the expression of Pro-EK-NES1 and Pro-EK-NES1-Flag in insect and mammalian cells. In each instance, purified plasmid DNA (5 μg) was transfected in MDA-MB-231 or Sf21 cells as described earlier and recombinant Pro-EK-NES1 and Pro-EK-NES1-Flag were recovered in the conditioned media as soluble proteins.

All recombinant proteins were purified as described above. The recombinant pro-EK-NES1-Flag was purified by one step affinity chromatography using anti-Flag-monoclonal antibody. Interestingly, when assessed by SDS-PAGE both recombinant proteins decreased in size by ~8 kDa after treatment with EK, although the expected size difference should be about 1 kDa (Fig. 29). A possible explanation is a potential glycosylation site at N 39 and with EK treatment the glycosylation chains are also removed. Amino acid sequence analysis revealed that, after treatment with EK both recombinant proteins possessed an N-terminal sequence of LDPEAY, which is identical to that of mature NES1 deduced from its cDNA (Fig. 29). For sequence of NES1, please see Appendix Ms. # 1.

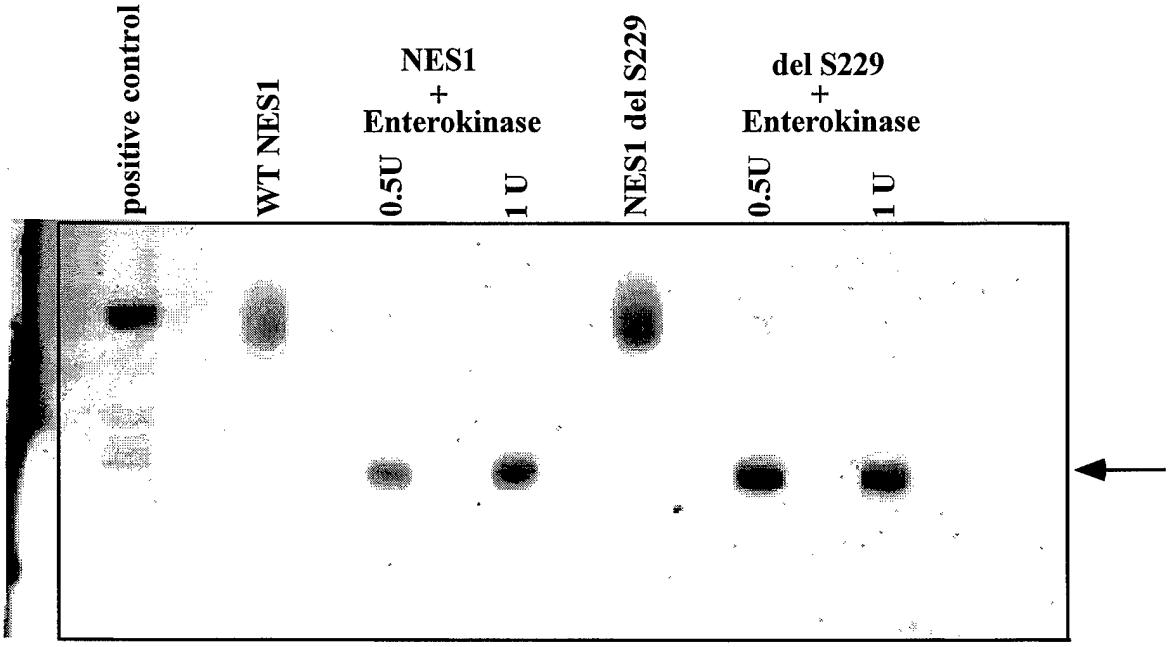


Fig. 29. Conversion of pro-NES1 to mature NES1 by Enterokinase. Purified NES1 protein with enterokinase recognition sequence DDDDK at the predicted cleavage site R42 was treated with recombinant enterokinase for 16 hours at room temperature, reaction products were separated on a 17% SDS-PAGE and stained with silver staining. Arrow indicates the mature form of NES1 as confirmed by N-terminal sequencing (data not shown).

These preparations were then examined for protease activity as described above. Data are shown below in Fig. 30 and 31.

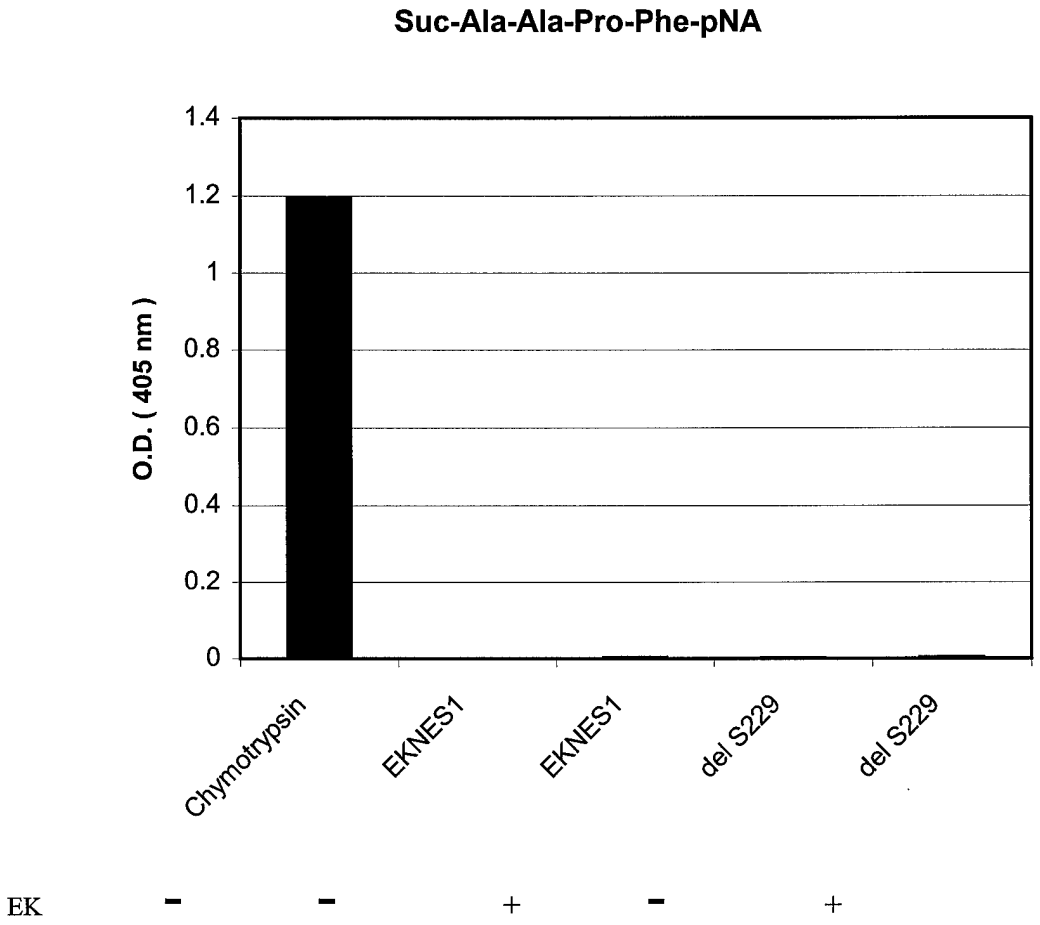


Fig. 30. Proteolysis of substrate Suc-Ala-Ala-Pro-Phe-pNA by EKNES1 and EKNES1-ΔS229: 5 μg each of purified proteins of EKNES1 or EKNES1-del229 were treated with recombinant enterokinase for 16 hours at room temperature to remove pro-sequences of NES1, substrate was then added to the reaction mixture and the release of paranitroaniline was measured at 405 nm. 400 ng of chymotrypsin was used as a positive control. – and + indicate absence or presence of EK.

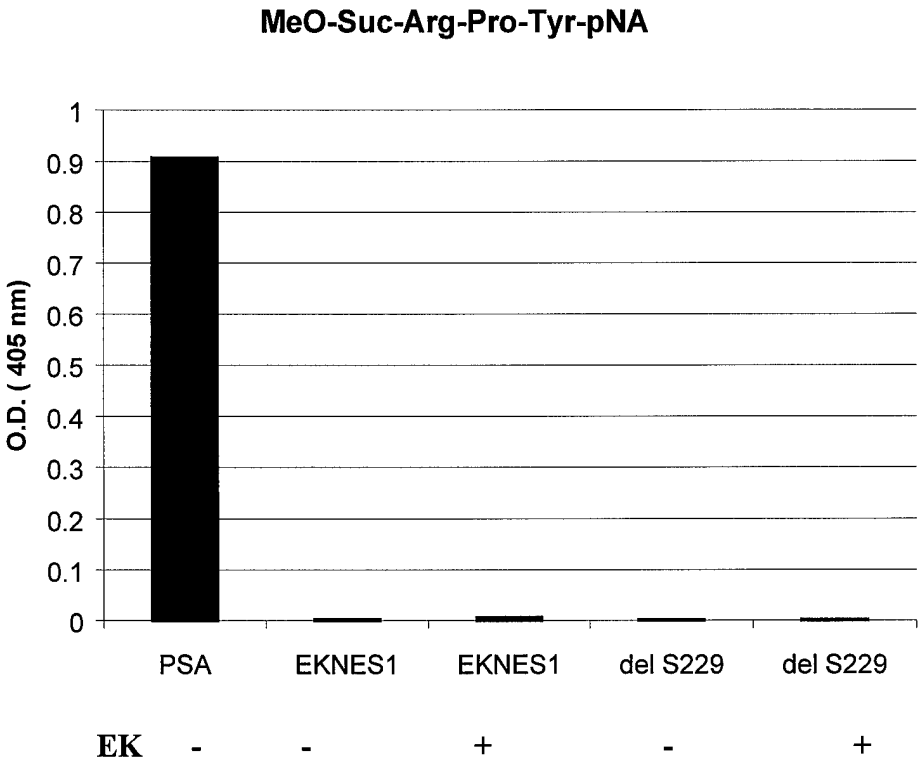


Fig. 31. Proteolysis of substrate MeO-Suc-Arg-Pro-Tyr -pNA by EKNES1 and EKNES1-del229: 5 μg of purified EKNES1 or EKNES1-del229 were treated with recombinant Enterokinase for 16 hours as above, substrate was then added to the reaction mixture and the release of paranitroaniline was measured at 405 nm. 5 μg of PSA was used as a positive control.

Summary of the NES1 expression, purification and protease activity.

The full length NES1 cDNA and two different mutants of predicted active serine residues (del229 and S229A) of NES1 were either expressed in insect cells or MDA-MB-231, a breast cancer cell line. Immunodetection revealed that NES1 was a secreted protein. The secreted wild-type and mutant NES1 proteins were purified from conditioned media by a combination of SP-Sepharose ion exchange and Bio-gel P-60 gel filtration chromatography. N-terminal sequencing demonstrated that NES1 was secreted as pro-NES1, having been cleaved of the predicted signal peptide (amino acids 1-33), with an intact pro-peptide preceding the putative cleavage site (R42). The pro-NES1 had no enzymatic activity. Efforts to activate NES1 by using limited proteolysis of pro-NES1 with low concentrations of trypsin, glandular kallikrein and also by changing the pH and temperature of protease reactions, failed to show any protease activity of NES1 on a universal serine protease substrate, casein resourfin and on a number of synthetic peptide substrates. These results suggested that either NES1 is an inactive protease or we are unable to remove pro-sequences. Therefore, we generated recombinant wild-type and mutant NES1 with enterokinase (Ek) cleavage site at R42, that could be activated after its purification. Ek-NES1

was purified by conventional chromatography or by affinity chromatography using Flag-affinity column (for FLAG-tagged NES1). After purification and Ek treatment we showed that wild-type as well as mutant NES1 was cleaved at R42 and contained expected amino terminus starting from LDPEAY. EK- treated wild-type and mutant NES1 proteins were then tested for enzymatic activity by using peptide substrates. Inspite of all our efforts, we could not demonstrate any protease activity in NES1. We conclude that NES1 lacks protease activity. A probable cause may be the unusual N-terminus of NES1 cleaved from Pro sequences. Interestingly, most of active serine proteases have a N-terminus usually starting with isoleucine (most common amino terminus IVGG) which is important in making the salt bridge after removal of pro-piece and the assembly of the catalytic triad rendering the activity to the protease. Salt bridge formation between Ile-Asp residues is considered the primary switch mechanism for activating protease. NES1 on the other hand has a sequence of LDPEAY (See NES1 sequence in attached Appendix Ms. # 1) and that may be the reason for NES1 to not act as an active protease.

KEY RESEARCH ACCOMPLISHMENTS

In this study we demonstrate:

1. NES1 mRNA/protein is expressed in normal cells but not in tumor cells.
2. NES1 mRNA/protein is down-regulated in breast, prostate and cervical cancer cell lines.
3. NES1 gene is localized to chromosome 19q13.3.
4. NES1 is synthesized as Pre-pro-NES1 and secreted as a pro-NES1 protein.
5. NES1 lacks protease activity. A probable cause may be the unusual N-terminus of NES1 LDPEAY instead of IVGG present in most active serine proteases.
6. Overexpression of NES1 leads to inhibition of tumorigenic phenotype (decreased anchorage independent growth and decreased tumorigenicity.
5. Overexpression of NES1 protein in an NES1-negative cell line causes decreased branching on matrigel.
6. NES1 promoter linked to luciferase reporter showed promoter activity.
7. Downregulation of NES1 in tumor cells is not due to decreased promoter activity.
8. Hypermethylation of NES1 gene is responsible for downregulation of NES1 in tumor cells.
9. Exon 3 is the site for NES1 methylation.
10. A strict correlation between exon 3 hypermethylation and loss of NES1 expression in a panel of breast and cervical cancer cell lines.
11. Importantly, using primary breast tumor samples, we demonstrate that hypermethylation of exon 3 correlates with lack of NES1 expression.
12. Treatment of NES1-nonexpressing cells with a demethylating agent led to re-expression of NES1.
13. Using In-situ hybridization technique with sense and anti-sense NES1 probe, we show abundant NES1 expression in normal tissue and complete loss in six out of six infiltrating ductal carcinomas.

REPORTABLE OUTCOMES:

Liu X-L, Wazer DE, Watanabe K, and Band V. Identification of a novel serine protease-like gene, the expression of which is down-regulated during breast cancer progression. Cancer Res., 56:3371-3379, 1996.

Goyal J, Smith KM, Cowan JM, Wazer DE, Lee SW, and Band V. The role for NES1 serine protease as a novel tumor suppressor. Cancer Res., 58:4782-4786, 1998.

J Goyal, K M Smith, J W Cowan, D E Wazer, S W Lee and V Band. The role of NES1 serine protease as a novel tumor suppressor. Poster presented by Dr. Goyal at the American Association for Cancer Research, 90th annual meeting, Philadelphia, PA. Proceeding of Am. Asso. Cancer Res. Volume 40, p35, 1999.

J Goyal, S Dhar, D E Wazer and V Band. NES1, a novel serine protease inhibits tumorigenic phenotypes of breast cancer cells. Poster presented by Dr. Goyal at the Era of Hope, DOD, 3rd annual meeting, Atlanta, GA, June 2000.

B Li, J Goyal, S Dhar, G Dimri, E Evron, S Sukumar, D Wazer and V Band. CpG methylation in exon 3 as a basis for breast tumor-specific loss of NES1 expression. Manuscript in Preparation (Once in Press, will be forwarded to DOD)

J Goyal, S Dhar, B Li, D E Wazer and V Band. Mutational analysis of the putative serine protease NES1 demonstrates that NES1 exhibits no proteolytic activity. Manuscript in Preparation (Once in Press, will be forwarded to DOD)

Cell lines: We have generated NES1-expressing breast cancer cell lines (MDA-MB-231, MDA-MB-468 and MDA-MB-435). We have also expressed NES1 in insect cells.

Reagents: We have cloned full-length NES1 cDNA, NES1-promoter, NES1CMVneo, NES1 cDNA in insect vector, methylation specific probes.

PATENT: A patent was filed before the grant was submitted. Now the patent is granted. -U.S. Patent No. 5,843,694
Methods for the identification of modulatory compounds for the expression of the NES1 protein.

All Personnel received salary from this grant:

Vimla Band, Ph.D.
Sanjay Dhar, Ph.D.
Jaya Goyal, Ph.D.
Biao Li, M.D., Ph.D.
Seetha Srinivasan, M.S.

CONCLUSIONS

NES1 (Normal Epithelial Cell Specific-1) gene is expressed in normal mammary epithelial (MECs) and prostate cells, but its mRNA and protein expression is dramatically decreased in most established breast, prostate and cervical cancer cell lines, even though no major deletion or rearrangements in NES1 gene are found. NES1 is expressed in several organs (thymus, prostate, testis, ovary, small intestine, colon, heart, lung and pancreas) with highest levels in ovary.

NES1 protein showed high homology to serine proteases. Expression and purification of a full-length NES1 cDNA and a serine mutant of NES1 in MDA-MB-231 cell line, showed that NES1 is synthesized as Pre-pro-NES1 in cells and is secreted as a pro-NES1 protein. The pro-NES1 had no enzymatic activity even after limited proteolysis by trypsin, glandular kallikrein and also by changing the pH and temperature of protease reactions. The protease activity of NES1 was tested by the proteolysis of the universal serine protease substrate, casein resorufin and a number of synthetic peptide substrates. Further use of recombinant wild-type and mutant NES1 with enterokinase (Ek) cleavage site at R42 EK-NES1, that could be activated after its purification, we showed that wild-type as well as mutant NES1 was cleaved at R42 and contained amino terminus starting from LDPEAY. EK- treated wild-type NES1 showed no enzymatic activity by using peptide substrates. Using S-minus mutant, we demonstrate that the initial observation that glandular kallikrein activated NES1 had some activity was somehow an effect of kallikrein rather than NES1. Inspite of extensive efforts, we could not demonstrate any protease activity in NES1. A probable cause may be the unusual N-terminus LDPEAY of NES1 after removing Pro sequences. In contrast to NES1, most active serine proteases have an N-terminus usually starting with isoleucine (most common amino terminus IVGG) which is important in making the salt bridge after removal of pro-piece and the assembly of the catalytic triad rendering the activity to the protease. Salt bridge formation between Ile-Asp residues is considered the primary switch mechanism for activating protease.

Transfection of NES1 cDNA into an NES1-negative MDA-MB-231 breast cancer cell line suppressed the oncogenicity as revealed by inhibition of the anchorage-independent growth and tumor formation in nude mice. Fluorescence in-situ hybridization localized the NES1 gene to chromosome 19q13.3, a region that contains genes for related proteases, including the prostate specific antigen, and is rearranged in human cancers. The potential tumor suppressor role of NES1 was further supported by the observation that NES1 reconstitution led to inhibition of cells branching on matrigel. Based on these data we are currently analyzing the potential intracellular role of NES1.

Cloning of NES1 promoter and analyses of its activity showed that most tumor cell lines were able to support full or partial transcription from NES1 promoter, suggesting a role of cis-acting mechanisms for loss of NES1 expression in tumor cells. We show that hypermethylation

of NES1 gene represents one such mechanism. While NES1 promoter is CpG-poor, exons 2 to 4 are CpG rich, with a high density of these sequences within exon 3. Using PCR and sequence analysis of sodium bisulfite-treated genomic DNA, and PCR with methylation-specific primers, we demonstrate a strict correlation between exon 3 hypermethylation and loss of NES1 expression in a panel of breast and cervical cancer cell lines as well as in primary breast tumor samples. Treatment of NES1-nonexpressing cells with a de-methylating agent led to re-expression of NES1 mRNA, suggesting a potentially important role of hypermethylation in loss of NES1 expression. We suggest that loss of NES1 expression and hypermethylation can serve as tumor markers in breast cancer and possibly in other cancers.

In summary, we accomplished most of the tasks in Aim I, II and III. In addition, we defined the mechanism of loss of NES1 expression in tumor cells. Future studies should focus on analyses to evaluate NES1 expression/hypermethylation as potential marker for diagnosis and/or prognosis of breast and other cancers. Also, we will make efforts to identify mechanisms involved in non-protease dependent tumor suppressor function of NES1.

[CANCER RESEARCH 56, 3371–3379, July 15, 1996]

Identification of a Novel Serine Protease-like Gene, the Expression of Which Is Down-Regulated during Breast Cancer Progression[1]

Xiao-Long Liu, David E. Wazer, Kiyoko Watanabe, and Vimla Band[2]

Department of Radiation Oncology, New England Medical Center [X-L. L., D. E. W., K. W., V. B.], and Department of Biochemistry, Tufts University School of Medicine [V. B.], Boston, Massachusetts 02111

ABSTRACT

In an effort to isolate genes with down-regulated expression at the mRNA level during oncogenic transformation of human mammary epithelial cells (MECs), we performed subtractive hybridization between normal MEC strain 76N and its radiation-transformed tumorigenic derivative 76R-30. Here, we report the isolation of cDNA clones corresponding to a 1.4-kb mRNA species that is abundantly expressed in 76N cells but is drastically reduced in 76R-30 cells. Based on its selective expression in MECs compared with fibroblasts, the corresponding gene is designated *NES1* (normal epithelial cell-specific 1). Sequence analysis of the full-length NES1 cDNA clones revealed it to be a novel gene with a predicted polypeptide of 30.14 kilodaltons; *in vitro* transcription and translation confirmed this prediction. Database searches revealed a 50–63% similarity and 34–42% identity with several families of serine proteases, in particular the trypsin-like proteases, members of the glandular kallikrein family (including prostate-specific antigen, nerve growth factor γ, and epidermal growth factor-binding protein) and the activators for the kringle family proteins (including the human tissue plasminogen activator and human hepatocyte growth factor activator). Importantly, all of the residues known to be crucial for substrate binding, specificity, and catalysis by the serine proteases are conserved in the predicted NES1 protein, suggesting that it may be a protease. An antipeptide antibody directed against a unique region of the NES1 protein (amino acids 120–137) detected a specific 30-kilodalton polypeptide almost exclusively in the supernatant of the mRNA-positive MECs, suggesting that NES1 is a secreted protein. The 1.4-kb NES1 mRNA was expressed in several organs (thymus, prostate, testis, ovary, small intestine, colon, heart, lung, and pancreas) with highest levels in the ovary; a 1.1-kb transcript was found in the pancreas. Although expression of the NES1 mRNA was observed in all normal and immortalized nontumorigenic MECs, the majority of human breast cancer cell lines showed a drastic reduction or a complete lack of its expression. The structural similarity of NES1 to polypeptides known to regulate growth factor activity and a negative correlation of NES1 expression with breast oncogenesis suggest a direct or indirect role for this novel protease-like gene product in the suppression of tumorigenesis.

INTRODUCTION

Breast cancer is the second leading cause of cancer-related deaths of women in North America and Europe. About 180,000 new cases of breast cancer are diagnosed every year in the United States alone, with one in eight women estimated to develop breast cancer during her lifetime. In nearly all cases, the etiology of these cancers is unknown. Similar to other human cancers, the vast majority of breast cancers represents a malignant transformation of epithelial cells. Malignant transformation represents a complex, multistep process in which genetic changes and environmental factors, such as radiation, viruses, and dietary factors, are thought to deregulate the cellular processes that control cell proliferation and differentiation (1). Therefore, identification and characterization of cellular genes that are targeted by the oncogenic stimuli and, in particular, the potential roles of epithelial cell-specific components is likely to enhance our understanding of the molecular basis of breast cancer.

Recent molecular studies have focussed on genetic lesions, such as deletions, mutations, and amplification of genes, involved in the control of cell growth. These studies have identified the important roles of tumor suppressor, growth factor, growth factor receptor, and proto-oncogene products in the genesis of cancers (2). In an attempt to define novel genes that are likely to play a role in breast and perhaps other epithelial cancers, we have recently established an *in vitro* model of MEC[3] oncogenesis. Based on the epidemiological evidence of the role of radiation in breast cancer (3–5), we exposed a normal MEC strain to fractionated doses of γ-irradiation, which led to its tumorigenic transformation (6). The pair of normal (76N) and radiation-transformed tumorigenic (76R-30) MECs provided a novel system to identify genes with specifically altered expression during oncogenesis. Indeed, initial studies demonstrated a specific loss of *p53* tumor suppressor gene expression in 76R-30 cells as a result of the deletion of one allele and a small deletion in the second intronic allele, which caused exon skipping (6).

Here, we used subtractive hybridization between the 76R-30 and 76N cells to isolate a novel cDNA, designated NES1 (for normal epithelial cell specific 1), the mRNA expression of which is dramatically down-regulated in radiation-transformed 76R-30 cells and absent in most of established breast cancer cell lines. NES1 shows a high homology with the families of members of three serine proteases: the trypsin-like family, kallikrein family, and activators of kringle domain-containing growth factors (7). Several of the serine proteases that show homology with NES1, such as mouse nerve factor γ, mouse EGF-binding protein, human tissue plasminogen activator, and hepatocyte growth factor activators, are known to convert inactive precursors of growth factors to mature, active growth factors (8–12). The structural similarity of NES1 with cell growth regulatory proteases and down-regulation of its mRNA expression in breast cancer cells suggest a potential role of this novel protein in the maintenance of the untransformed state of mammary and perhaps other epithelial cells.

MATERIALS AND METHODS

Sources of Cells. Normal mammary epithelial cells (81N and 76N), fibroblast cells (76NF and 81NF), radiation-transformed cells (76R-30), HPV-16 E6 and/or E7-immortalized epithelial cells (81E6, 76E7, 81E7, 76E6E7, and 7VNE6E7), and milk-derived epithelial cells-immortalized with HPV-16 E6 and E7 (ME6E7, M2E6E7, M3E6E7, and M4E6E7) were established in our laboratory (6, 13, 14). The MCF-7 cell line was obtained from the Michigan Cancer Foundation. All other breast tumor cell lines (MDA-MB-134, MDA-MB-157, MDA-MB-175, MDA-MB-231, MDA-MB-361, MDA-MB-415, MDA-MB-435C, MDA-MB-436, MDA-MB-453, BT-474, BT-483, BT-549, ZR-75-1, ZR-75-30, T-47D, and SK-BR-3) were obtained from the American Type Culture Collection.

Received 3/7/96; accepted 5/16/96.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by departmental start-up funds (V. B.).

[2] To whom requests for reprints should be addressed, at Department of Biochemistry, Tufts University School of Medicine, 750 Washington Street, Boston, MA 02111. Phone: (617) 636-4776; Fax: (617) 636-6205.

[3] The abbreviations used are: MEC, mammary epithelial cell; HPV, human papillomavirus; poly(A)+, polyadenylated; oligo(dT), oligodeoxythymidylic acid; kDa, kilodalton; NGF, nerve growth factor; EGF, epidermal growth factor.

Cell Culture Conditions. Normal and immortal mammary epithelial cells were maintained in DFCI-1 medium (13). Immortalized milk-derived epithelial cells were grown in DFCI-1 medium containing 10% FCS (14). All tumor cell lines were grown in α-MEM supplemented with 10% FCS, as described earlier (15).

Construction of cDNA Libraries. A subtraction cDNA library was custom made through Novagen (Madison, WI). Briefly, a cDNA library was first prepared by reverse transcription from 76R-30 poly(A)$^+$ RNA using an oligo(dT)-*Hin*dIII primer adapter, klenow-mediated second-strand cDNA synthesis, attachment of *Eco*RI linkers, and directional cloning into *Eco*RI-*Hin*dIII sites of the λSHlox vector (16). Phage DNA was prepared from the 76R-30 cDNA library, digested with *Not*I, and *in vitro* transcribed with the T7 RNA polymerase to yield the "driver" cRNA for subtraction. cRNA was biotinylated using photoprobe biotin (17). For subtraction, ^{32}P-labeled first-strand cDNA was prepared from 76N poly(A)$^+$ RNA by reverse transcription. This cDNA was subjected to two rounds of subtractive hybridization with a 10-fold excess of biotinylated 76R-30 driver cRNA (16, 17). Hybridization was prepared in the presence of poly(A)$^+$ oligomers to prevent oligo(dT)/oligo(dA) interactions between the cRNA and cDNA. After each round, excess driver cRNA and double-stranded hybrids were removed with streptavidin and phenol. This procedure resulted in the depletion of 99.54% of 76N cDNA. The remaining single-stranded 76N cDNA was used for second-strand cDNA synthesis using Klenow; *Eco*RI adapters were ligated to ends; and cDNA was cloned into the *Eco*RI and *Hin*dIII sites of the λSHlox vector. This cDNA library is referred to as the 76NS library. Recombinant phages were plated at low density, and single plaques were picked and converted into the plasmid form by Cre-recombinase-mediated autosubcloning (16). The plasmid DNA was isolated from individual colonies and digested with *Eco*RI and *Hin*dIII. Isolated *Eco*RI-*Hin*dIII inserts were ^{32}P labeled and used as probes for Northern blot hybridization to poly(A)$^+$ mRNA isolated from the 76N and 76R-30 cells.

A second 76N cDNA library in the pGAD10 vector was custom constructed through Clontech (Palo Alto, CA). Here, the first-strand cDNA was synthesized using oligo(dT) plus random primers, followed by DNA polymerase 1-mediated second-strand synthesis, addition of *Eco*RI adapters, and cloning into the *Eco*RI site of the PGAD10 vector (18). This library was screened with ^{32}P end-labeled oligonucleotides (see "Results").

Sequencing of the *NES1* Gene. pSHlox-1 plasmid recombinants containing NES1 cDNA inserts were used as templates for double-strand sequencing using Sequenase (United State Biochemical Corp., Cleveland, OH). Sequences near the ends of cDNA inserts were obtained with the following primers corresponding to flanking the vector sequences. pSHlox vector primers were: T7 promoter primer, 5′-GGC-CTC-TAA-TAC-GAC-TCA-C-3′; and SP6 promoter primer, 5′-CCG-CAG-ATT-TAG-GTG-ACA-C-3′. PGAD10 primers were: an upstream primer, 5′-TAC-CAC-TAC-AAT-GGA-TG3′; and a downstream primer, 5′-GTT-GAA-GTG-AAC-TTG-CGG-GC3′. The remaining part of the NES1 inserts was sequenced using 12 sense strand (nucleotides 6–22, 72–91, 128–145, 196–213, 344–360, 484–500, 634–650, 723–739, 851–867, 998-1116, 1125–1141, and 1253–1269) and 10 antisense strand (nucleotides 1392–1377, 1294–1277, 1201–1185, 1086–1069, 917–899, 807–789, 674–657, 516–488, 292–275, and 176–161) NES1-specific primers.

***In Vitro* Translation of NES1.** An NES1 cDNA fragment containing nucleotides 1–1069 (see Fig. 2), which include the predicted polypeptide-coding region, was cloned in the pBluescript II KS vector (Stratagene, La Jolla, CA) in both sense and antisense orientations, and plasmid DNA was used for coupled *in vitro* transcription and translation, using a commercial kit (Promega, Madison, WI), as described (19). [^{35}S]methionine was incorporated during translation to allow visualization of the synthesized polypeptide by SDS-PAGE followed by fluorography.

RNA Isolation and Northern Blot Analysis. Total cellular RNA was isolated from 50–70% confluent cell monolayers using the guanidium-isothiocyanate method (20). Northern blot hybridization was carried out using a nylon membrane (Hybond-N; Amersham, Arlington Heights, IL) and ^{32}P-labeled cDNA insert (nucleotides 651-1072), as described previously (21). Nylon membranes with poly(A)$^+$ RNAs from various human tissues (tissue blots) were obtained from Clontech.

Southern Blot Analysis. Ten μg genomic DNA were digested to completion with restriction enzymes (Life Technologies, Inc., Gaithersburg, MD), resolved on agarose gels, transferred to a Hybond-N nylon membrane, and hybridized with a ^{32}P-labeled NES1 cDNA probe corresponding to nucleotides 1–1069, as described earlier (21).

Generation of Anti-NES1 Antibody. A NES1 peptide (amino acids 120–137, n..C-K-Y-H-Q-G-S-G-P-I-L-P-R-R-T-D-E-H-D..c) was coupled to keyhole limpet hemocyanin through a C-terminal cysteine residue using maleimidobenzoyl-*N*-hydroxysuccinimide ester (Pierce Chemical Co., Rockford, IL) for chemical cross-linking. The conjugate was used to immunize rabbits as a 1:1 emulsion with Titermax adjuvant (CytRx Corp., Norcross, Georgia), as described earlier (22). Booster injections were given every 30 days, and rabbits were bled 10 days after each injection.

Western Blot Analysis. Normal, immortalized, and tumor mammary cells were grown to 80–90% confluency in their respective media in 25-cm^2 flasks. Cells were then washed once with a serum and bovine pituitary extract-depleted DFCI-1 medium (D2) as described earlier (21) and then further cultured in this medium for 20 h. Supernatants were collected, and cells were lysed in SDS-PAGE sample buffer. The supernatant from each flask was concentrated to 150 μl using a 10-kDa cutoff filter (ultrafree centrifugal filter device; Millipore, Marlborough, MA) and mixed with an equal amount of 2× sample buffer. Concentrated supernatant and lysates representing an equal number of cells were resolved on 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore) as described earlier (14). Membranes were incubated with anti-NES1 antisera followed by goat antirabbit IgG conjugated to horseradish peroxidase (Pierce). Enhanced chemiluminescence detection was performed according to the manufacturer's instructions (Amersham).

RESULTS

Isolation of a cDNA Clone Corresponding to an mRNA Expressed in 76N Cells but Markedly Down-Regulated in the 76R-30 Radiation-transformed Cell Line. A cDNA library was prepared from the poly(A)$^+$ RNA of the 76N normal MEC strain after the RNA messages cross-hybridizing with its radiation-transformed derivative 76R-30 strain (6) were depleted by two rounds of subtractive hybridization (see "Materials and Methods"). This resulted in the removal of 99.54% of the total cDNAs, suggesting that the remaining cDNAs were likely to be enriched for the mRNA species that are preferentially expressed in the 76N cells. Single bacteriophage plaques from this library (76NS) were converted into plasmid form by Cre-mediated recombination, and their cDNA inserts were cleaved with *Eco*RI and *Hin*dIII (restriction enzymes used for cloning; Refs. 16 and 17). Isolated cDNA inserts were labeled with [^{32}P]dCTP and [^{32}P]dGTP and used as probes in Northern blotting to detect the relative mRNA expression in 76N and 76R-30 cells. One cDNA insert (clone 37, about 0.4 kb) hybridized to an abundant 1.4-kb transcript in 76N normal parental cells (Fig. 1). In contrast, 76R-30 cells showed dramatically reduced expression of this mRNA. Thus, clone 37 represented a gene with expression that appeared to be down-regulated at the mRNA level during oncogenic transformation of the 76N MECs. As described below, mRNA corresponding to cDNA clone 37 is expressed in MECs but not in mammary fibroblasts. Therefore, the corresponding gene has been designated normal epithelial cell-specific 1 or *NES1*.

Sequence Analysis of NES1 cDNA Reveals It to Be a Novel Serine Protease-like Gene. The initial NES1 cDNA insert in the pSHlox-1 plasmid was sequenced from both ends using oligonucleotide primers corresponding to flanking SP6 and T7 promoters. Comparison of this sequence (418 bp) with published sequences (GenBank) revealed no matches, indicating that *NES1* represented a novel gene. To obtain full-length cDNA clones, the initial 418-bp NES1 cDNA was used as a probe to isolate additional cDNA clones from the 76NS λSHlox cDNA library. In addition, a second 76N cDNA library containing random- and oligo(dT)-primed cDNAs in the pGAD10 vector was screened with a NES1 oligonucleotide primer (nucleotides 674–657). The ends of the cDNA clones isolated by these two

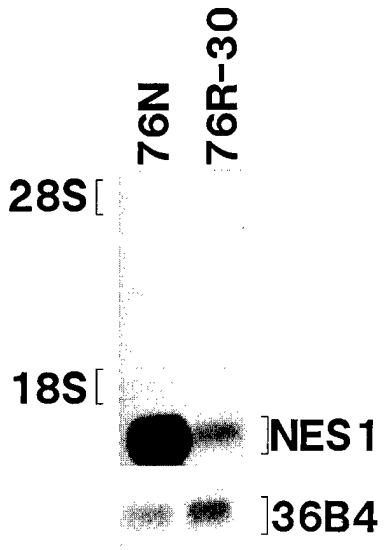


Fig. 1. Northern blot analysis of NES1 mRNA expression in 76N *versus* 76R-30 cells. Poly(A)$^+$ RNA from both cell lines was resolved on a 1.5% agarose-formaldehyde gel, transferred to a nylon membrane, and hybridized with a ^{32}P-labeled, 418-bp NES1 probe and visualized by autoradiography. Locations of the rRNAs (28S, 4850 bp; and 18S, 1740 bp) are indicated. 36B4 was used as a loading control.

approaches were sequenced using primers corresponding to flanking vector sequences. Further sequences were determined using oligonucleotide primers corresponding to determined NES1 sequences (see "Materials and Methods" for primers). Using this strategy, a sequence of 1454 nucleotides was determined from overlapping cDNA clones in both directions (Fig. 2).

Translation of the determined nucleotide sequence of the NES1 cDNA clones revealed a 276-amino acid open reading frame starting with the first methionine codon at nucleotides 82–84, which occurs in a context (GCCATGA) that is compatible with a consensus Kozak sequence (A/GCCATGG; Ref. 23). The use of a less favorable ATG (nucleotide 154–156) would result in a 252-amino acid polypeptide. Based on the results of *in vitro* translation (see below), we favor the possibility that nucleotides 82–84 serve as the initiation codon (Fig. 2). Although the exact transcription start site of *NES1* has not been determined, the close correspondence of the determined sequence (1454 nucleotides) with the NES1 mRNA size (1.4 kb) suggests that the sequences upstream of the first methionine represent the 5′ untranslated region of the mRNA. This suggestion is reinforced by the comparison of NES1 to other homologous proteins (see below). Nucleotides 1417–1422 (ACTAAA) closely resemble a consensus polyadenylation signal (AATAAA; Ref. 24) and are followed by a stretch of 14 adenines after a space of 17 nucleotides. No other potential polyadenylation signals were discernible in the 3′ untranslated region, suggesting that nucleotides 1417–1422 represent the polyadenylation signal (Fig. 2).

Although the NES1 sequence is unique, data base comparison with available sequences revealed it to be highly homologous to a number of serine proteases. When the entire predicted NES1 polypeptide was used for homology comparisons (Lipman-Pearson protein alignment method), it showed 34–42% identity and 50–63% overall similarity with members of three distinct families of serine proteases: the trypsin family, the kallikrein family, and serine proteases that activate the kringle domain-containing growth factors (Fig. 3 and Table 1). Notably, the sequence identity was highest around the residues that are known to be involved in substrate binding and specificity and catalytic activity of the serine proteases (Figs. 3 and 4). In particular, all of the three residues (serine 231, aspartic acid 137, and histidine 86) that form the catalytic triad of serine proteases were found in relative locations similar to those of other serine protease (Fig. 4).

Sequence alignment also revealed a potential cleavage site (Arginine 42) at a site homologous to other serine proteases (Lysine or Arginine in most cases), suggesting that NES1 may be synthesized as a precursor (25). Additional similarities with serine proteases included a similar overall amino acid. length and 40–50% nucleotide sequence homology within the 5′ and 3′ untranslated regions. Altogether, these features strongly suggest that the *NES1* gene encodes a novel serine protease.

Interestingly, the kallikrein family serine proteases that showed the sequence homology to NES1 included prostate-specific antigen, the NGF 7S component, the NGF-γ component, tissue plasminogen activator, and EGF-binding protein (8–12, 26). Detailed analysis of NES1 did not reveal it to be closer to either the trypsin-like or kallikrein-like proteases, the members of which showed between 53 and 78% identity among themselves. The level of homology between trypsin and kallikrein family was similar to their homology with NES1 (30–40% identity). Furthermore, several amino acid stretches in NES1 showed poor homology to other proteases. These include amino acids 44–48, 122–130, and 148–151, and other regions throughout the predicted polypeptide sequence. In addition, the putative signal peptide sequences (first 14 or 15 amino acids after methionine) were not closely related to the corresponding sequence in trypsin, EGF-binding protein, or human tissue plasminogen activator (Fig. 3). Interestingly, both the NES1 and kallikrein family proteins (such as EGF-binding protein) showed a small insert (NES1, amino acids 126–136) immediately before His86 of the catalytic triad; however, this region and the immediate NH_2-terminal sequences of NES1 were not particularly homologous to either the kallikrein or trypsin family proteases. Taken together, these features suggest that NES1 is a distinct serine protease.

Hydropathicity analysis (Fig. 5) revealed the NH_2-terminal region to be quite hydrophobic, consistent with the possibility that this region may harbor a signal sequence analogous to other serine proteases (Fig. 3). Although the likelihood of this possibility is supported by the secretion of the NES1 protein (see below), this region did not show a strong homology to signal sequences of other serine proteases. Several evenly distributed hydrophobic regions throughout the NES1 polypeptide are consistent with a globular protein similar to other serine proteases.

***In Vitro* Translation.** The longest open reading frame of the NES1 cDNA (nucleotides 82–912) predicted a polypeptide of 30.14 kDa, whereas the use of the second methionine (nucleotide 154–156) predicted a polypeptide of 27.6 kDa. To experimentally determine the size of the polypeptide encoded by the NES1 cDNA clone, nucleotides 1–1069, which contained the predicted coding region and both potential initiation codons, were cloned in the pBluescript II KS vector in both sense and antisense orientations. The plasmid DNA was used for *in vitro* transcription, followed by translation in the presence of [^{35}S]methionine. As shown in Fig. 6, a 30-kDa polypeptide closely corresponding to the size predicted by the use of first methionine was observed in the translation reaction of the sense but not the antisense NES1 cDNA.

Tissue-specific Expression of NES1 mRNA. To assess the tissue distribution of NES1 expression, Poly(A)$^+$ RNA samples from various human tissues were hybridized with a NES1 probe corresponding to nucleotides 1–1069 of the full-length NES1 cDNA. As shown in Fig. 7, differential expression of the *NES1* gene was observed in the tissues examined. Relatively abundant levels of the 1.4-kb mRNA transcript were observed in the prostate, testis, ovary, small intestine, colon, and lung, with highest levels in the ovary. The pancreas showed abundant expression of a shorter (1.1-kb) mRNA transcript. In com-

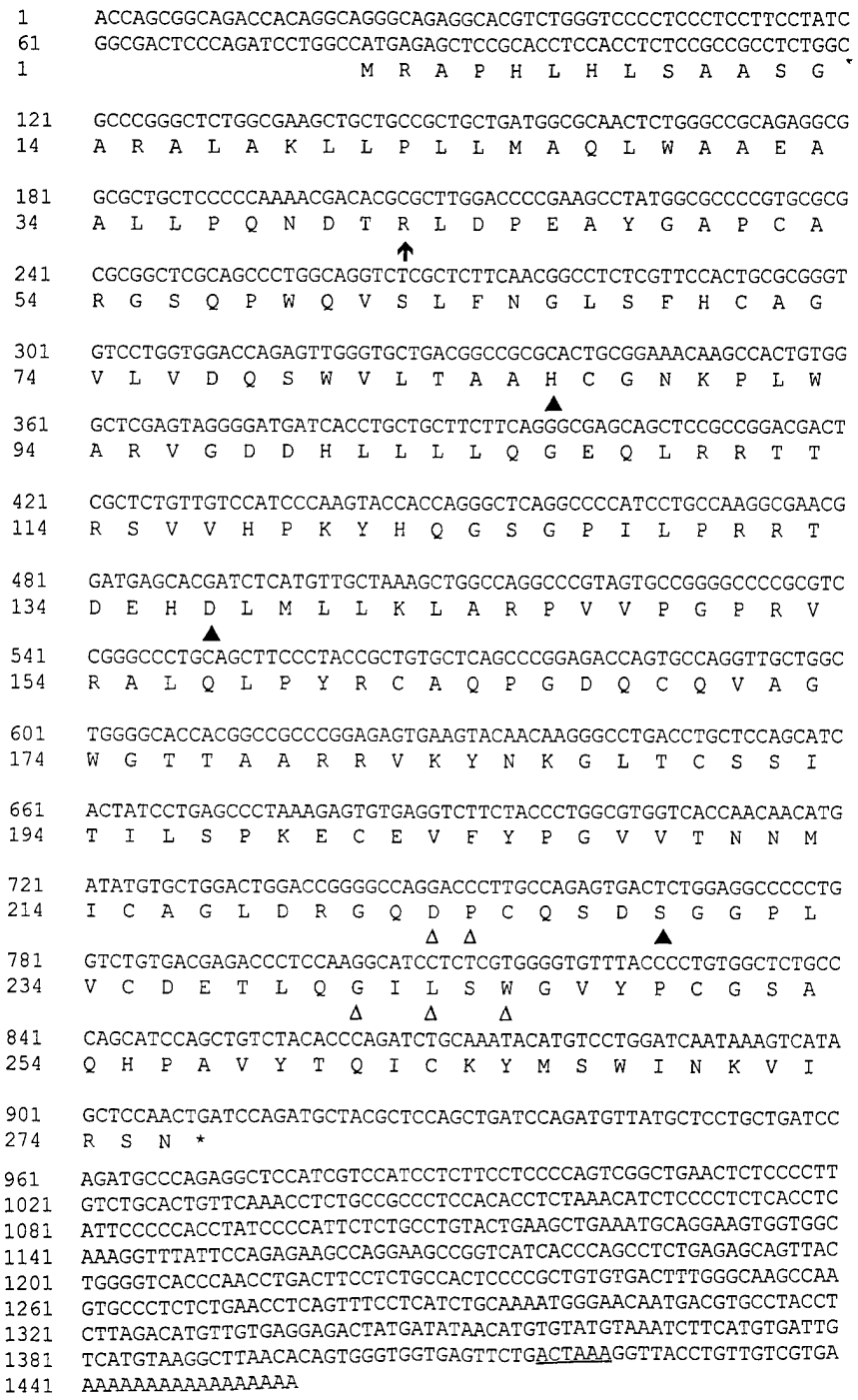


Fig. 2. Nucleotide sequence of the full-length *NES1* cDNA and the predicted amino acid sequence. Nucleotide sequence was obtained from multiple cDNA clones in both directions and is presented as a composite. ↑, cleavage site; ▲, catalytic triad; △, important amino acid in substrate binding; *, C terminus. Poly(A)$^+$ signal is *underlined*.

parison, NES1 expression in the thymus and heart was barely detectable and was essentially undetectable in peripheral blood leukocytes, brain, placenta, lung, liver, skeletal muscle, and kidney. Hybridization with the control probe 36B4 (27) demonstrated equal loading of mRNA in all lanes. These analyses show that NES1 mRNA is expressed in a large subset of human tissues.

Expression of *NES1* in Normal, Immortalized, and Tumor Mammary Cells. As described above, NES1 cDNA was isolated by virtue of its drastically reduced mRNA expression in MECs oncogenically transformed by γ-irradiation. To further assess the relationship between NES1 mRNA expression and mammary tumor progression, we analyzed a number of normal MECs, mammary fibroblasts, immortalized MECs, and established mammary tumor cell lines by Northern blot analysis. All of the normal (4 of 4) mammary epithelial cells expressed abundant levels of 1.4-kb NES1 mRNA, whereas none of the fibroblast cell strains (5 of 5) expressed detectable mRNA levels (hence, the designation normal epithelial cell specific 1 or NES1; Fig. 8 and data not shown). All of the MECs that have been induced to undergo preneoplastic transformation by HPV-16 E6 or E7 (10 of 10) expressed NES1 mRNA levels similar to those of normal MECs (Fig. 8 and data not shown). In contrast, a drastic decrease or complete loss of NES1 mRNA was observed in most (16 of 20) breast cancer cell lines examined (Fig. 8 and data not shown). Notably, all of the four breast cancer cell lines (21PT, 21NT, 21MT-1, and 21MT-2) that showed substantial NES1 mRNA expression (data not shown) are derived from a single breast cancer patient and are clonally related

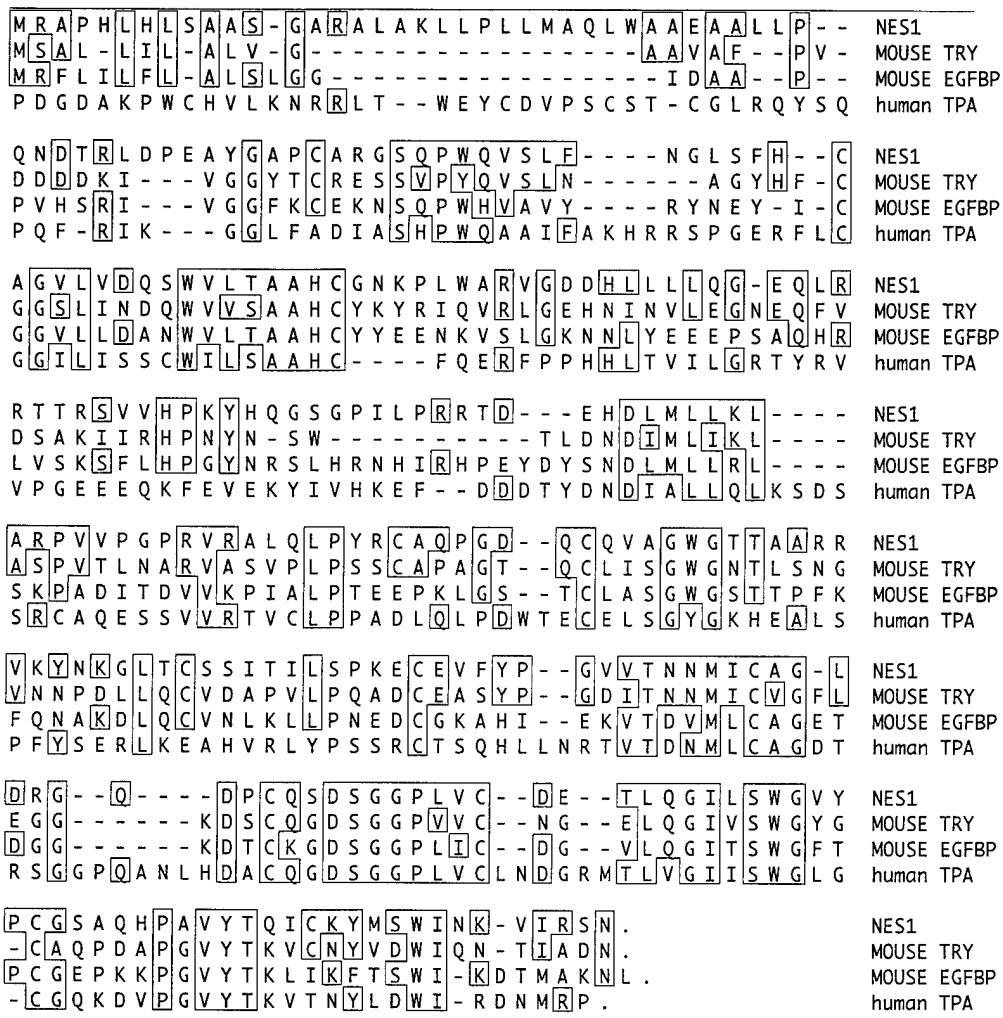


Fig. 3. Homology of the predicted NES1 amino acid sequences with serine proteases. Homology comparisons were made using the Clustal algorithm with a gap penalty of 3. Comparisons include NES1 amino acids 1–276, mouse preprotrypsin (*TRY*) amino acids 1–246, mouse EGF-binding protein (*EGFBP*) amino acids 1–261, and human tissue plasminogen activator (*TPA*) amino acids 270–562. ·, C terminus of the protein. Sequences in *boxes*, identity with the NES1 predicted amino acid sequence.

Table 1 *Amino acid homology of NES1 to other serine proteases*

Serine proteases	Similarity (%)	Identity (%)
Trypsin-like family		
Mouse preprotrypsin	63	42
Human pancreatic trypsinogen III	59	37
Human sapiens trypsinogen IV b	58	36
Kallikrein family		
Mouse nerve growth factor 7Sα	57	36
Mouse epidermal growth factor-binding protein	57	36
Mouse nerve growth factor γ	56	34
Human glandular kallikrein	54	38
Human kallikrein	53	35
Human prostrate specific antigen precursor	50	35
Kringle family		
Human tissue plasminogen activator	57	36
Human hepatocyte growth factor activator	53	34

(15). Reprobing of the blot with control probe 36B4 (27) showed that mRNA was indeed loaded in the lanes that showed little or no hybridization with the NES1 probe (Fig. 8). These results show that although NES1 mRNA expression is unaffected by immortalization (preneoplastic transformation) of mammary cells, it is drastically down-regulated in a large subset of established breast cancer cell lines.

Southern Blot Analysis of *NES1* Gene. To determine whether the decrease or loss of NES1 mRNA expression in oncogenically transformed cells was due to deletion or rearrangement of the gene, we performed Southern blot analyses of *Ban*II-, *Bgl*II-, or *Eco*RI-digested genomic DNA isolated from 76N, 76R-30, MCF-7, and MDA-MB-231 cells. As shown in Fig. 9, all four cell lines showed identical hybridizing bands of equal intensity. Therefore, loss of NES1 mRNA expression does not appear to be due to any major deletion or rearrangement of the gene. However, this analysis does not exclude the presence of more subtle mutations that may lead to loss of expression.

NES1 Is a Secreted Protein. Most of the proteins that showed homology to the predicted NES1 polypeptide contain a signal peptide at the NH_2 terminus (first 14–15 amino acids after methionine) and are secreted. Although the NH_2 terminus of NES1 did not show significant homology to the corresponding region of the other serine proteases, this region was strongly hydrophobic (Fig. 5), consistent with its being a signal peptide and suggesting that NES1 is a secreted protein. To examine this possibility, we generated a rabbit antiserum against a NES1-specific peptide (amino acids 120–137). This antiserum was used for Western blot analysis of cell lysates and culture supernatants derived from NES1 mRNA-positive and -negative MECs. As seen in Fig. 10, anti-NES1 antibodies specifically detected a 30-kDa polypeptide almost exclusively in the culture supernatants of all of the NES1 mRNA-positive cell lines. The 30-kDa polypeptide was not detected in the NES1 mRNA-negative cell lines. These data strongly support the possibility that NES1 is a secreted protein similar to other homologous serine proteases.

	86	137	223	224	227	228	229	230	231	241	243	245
NES1	H	D	D	P	S	D	S	G	G	G	L	W
Human Pancreatic Trypsinogen III	H ▲	D ▲	D △	S △	R	D	S ▲	G	G	G △	V △	W △

Fig. 4. Comparisons of putative active site and substrate-binding amino acids of NES1 with corresponding known residues in human trypsinogen III. ▲, catalytic triad and residues; △, residues important for substrate binding and specificity.

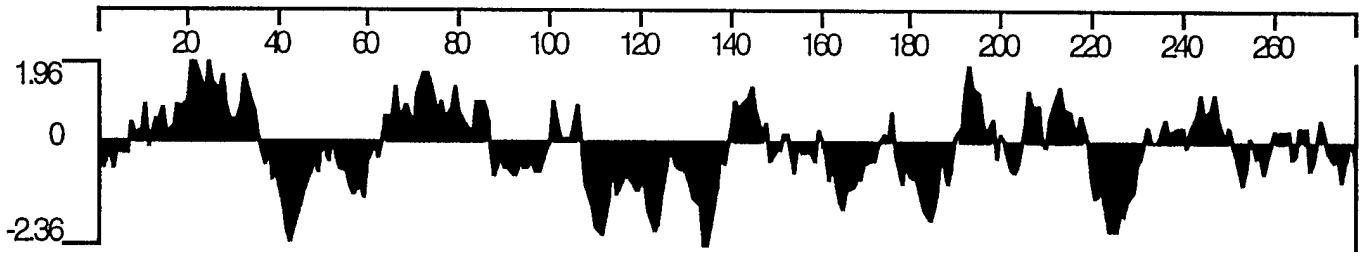


Fig. 5. Hydropathicity plot of the predicted NES1 polypeptide. Hydropathicity was calculated using the Kyte-Doolittle method in the DNASTAR program. Values greater than 0 represent hydrophobic regions, and those less than 0 represent hydrophilic regions.

DISCUSSION

Tumorigenesis represents a complex process in which altered expression of cellular genes plays an important role. Identification of these cellular genes is likely to enhance our understanding of the tumorigenic process and to provide diagnostic and/or therapeutic avenues in the future. Recently, we isolated a radiation-transformed tumorigenic derivative (76R-30) from normal human MEC strain 76N (6). This pair of cell lines provided a unique system to identify genes with altered expression during mammary tumorigenesis.

The present studies describe the isolation of a novel epithelial cell-specific cDNA, NES1, using subtractive hybridization between 76N and 76R-30 cells. As expected from its isolation from a subtracted cDNA library, the NES1 mRNA was abundantly expressed in 76N cells but was drastically reduced in 76R-30 cells. These analyses revealed a major mRNA species of 1.4 kb in 76N cells. Sequence analysis of the full-length cDNA clones showed that *NES1* is a novel gene. Data base comparison revealed that both the nucleotide and predicted amino acid sequences of *NES1* showed a strong homology (34–42% amino acid identity) to several distinct families of serine proteases, comparable to the level of homology between other protease families. In addition to sequence homology, all of the critical structural features of serine proteases were conserved in *NES1*. These include the invariant residues (histidine 86, aspartic acid 137, and serine 229) that form the catalytic triad of serine proteases (see Fig. 4 and Ref. 28). Additional residues surrounding serine 229, which are critical for substrate binding and specificity of serine proteases, are also conserved (Fig. 4). Compared with a 34–42% overall identity with known serine proteases, the sequences immediately around the critical catalytic center residues showed up to 95% identity with certain proteases. Finally, *NES1* also shows a potential tryptic cleavage site (Arginine 42) in a location similar to that seen in trypsinogen, at which it allows autocatalytic cleavage into active trypsin (25). Altogether, these conserved structural features strongly suggest that NES1 is a protease; however, this activity needs to be experimentally demonstrated.

Although the critical protease domains are conserved in NES1, it does show several unique structural features. Most notably, the NH_2 terminus of NES1 (which includes the putative signal peptide) and amino acids 44–48, 122–130, and 148–151 represent unique inserts not observed in other serine proteases. In addition, the level of homology between NES1 and other serine proteases (34–42% identity) is no more than the level of homology between other serine proteases belonging to distinct families, such as trypsin-like *versus* kallikrein-like proteins. In contrast, members of a single family typically showed 53–78% sequence identity. Thus, the divergence between NES1 and other serine proteases may have occurred early during evolution. This high level of divergence is consistent with unique biological functions of NES1.

Although the established physiological roles of the trypsin family of serine proteases are primarily in general protein catabolism (7), the functions of other serine proteases that share homology with NES1 are of special interest. For example, the NES1-related members of the glandular kallikrein family included NGF-γ and EGF-binding protein.

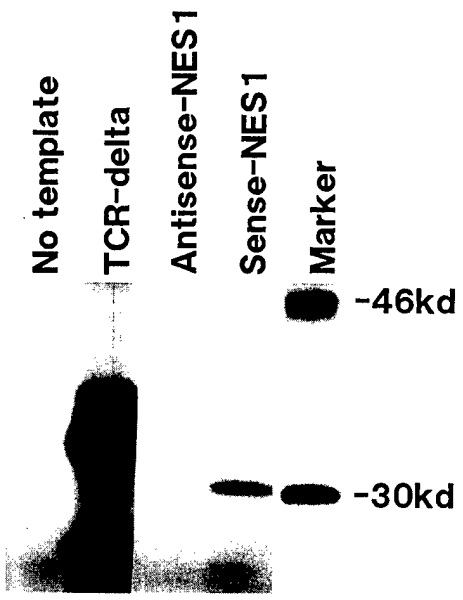


Fig. 6. *In vitro* translation of NES1 cDNA. NES1 cDNA fragment (1–1069 nucleotides) was cloned in pBluescript II KS vector in sense and antisense orientations, and plasmid DNA was used for *in vitro* translation using a coupled transcription-translation system in the presence of [^{35}S]methionine-cysteine (see "Materials and Methods"). ^{35}S-labeled, *in vitro*-translated products were run on a 10% SDS-PAGE. Note that sense but not antisense cDNA encoded a polypeptide of the predicted size (about 30 kDa). Human TCR-δ (expected size, 34 kDa; Ref. 47) was used as a positive control.

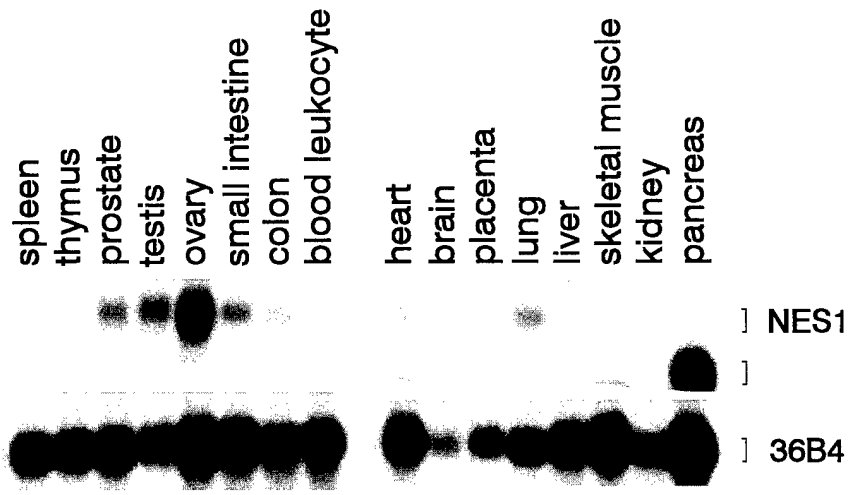


Fig. 7. Northern blot analysis of NES1 mRNA expression in various human tissues. Nylon membranes with 2 μg poly(A)$^+$ RNAs from each of the indicated human tissues were obtained from Clontech, hybridized with a ^{32}P-labeled NES1 probe, and visualized by autoradiography. 36B4 probe was used as a loading control.

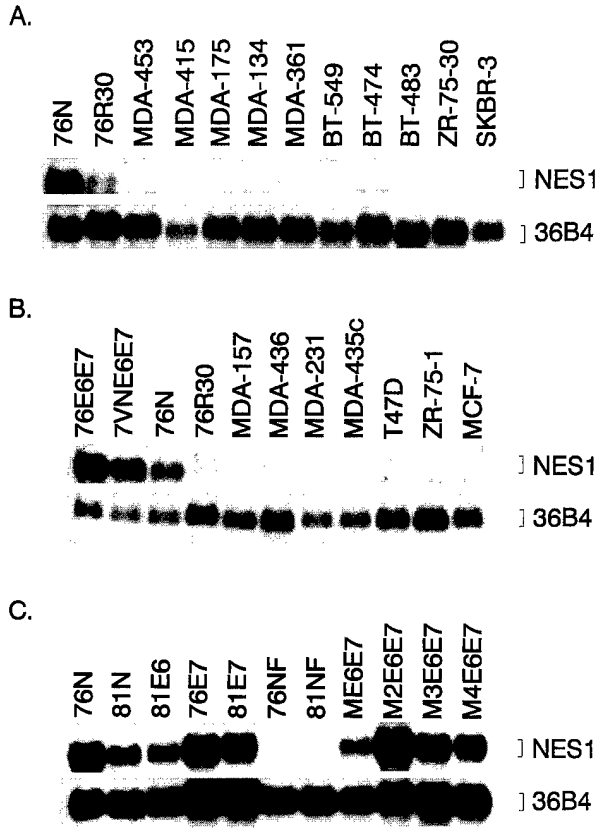


Fig. 8. Northern blot analysis of NES1 mRNA expression in cultured cell lines. Analysis was performed as described in Fig. 1 legend. Note a drastic decrease in NES1 mRNA levels in 76R-30 cells and almost complete loss in all mammary tumor cell lines. 36B4 was used as a loading control. *A–C* are separate blots. *A, 76N,* normal MEC; *76R-30,* radiation-transformed 76N MEC; remaining cell lines represent breast cancer-derived cell lines. *B, 76E6E7,* 76N cells immortalized by HPV-16 E6 and E7; *7VNE6E7,* 7VN cells immortalized by HPV-16 E6 and E7; *76N* and *76R-30,* as in *A;* remaining cell lines represent breast cancer-derived cell lines. *C, 76N* and *81N,* two normal mammary epithelial cell strains; *81E6,* HPV-16 E6-immortalized 81N cell; *76E7* and *81E7,* HPV-16 E7-immortalized 76N and 81N cells, respectively; *76NF* and *81NF,* two mammary fibroblast cell strains from two individuals designated 76 and 81, respectively; other cell lines are HPV-16 E6- and E7-immortalized human milk-derived epithelial cell lines from three separate specimens.

These proteins are known to proteolytically activate NGF-β and EGF, respectively (10, 29). NES1 also exhibited a similar level of homology to human prostate-specific glandular kallikrein, prostate-specific antigen and the pancreatic and renal kallikrein (26, 30, 31). All of these proteins are known to have protease activities against specific substrates, resulting in the liberation of a bioactive peptide or polypeptides (26, 30, 31). Finally, NES1 showed homology to human tissue plasminogen activator and the hepatocyte growth factor activator precursor (11, 12). These proteins are known to activate plasminogen and hepatocyte growth factor, respectively (11, 12). The latter proteins are members of the kringle protein family, characterized by the presence of a kringle domain (7). Significantly, hepatocyte growth factor binds to and activates a transmembrane receptor tyrosine kinase that regulates mitogenesis and differentiation of the target cells (32). These established activities of NES1 homologues raise the possibility that NES1 may serve to positively or negatively regulate cell growth or differentiation by cleavage of cell growth regulatory proteins.

NES1 cDNA was isolated by virtue of its decreased expression in a tumorigenic derivative (76R-30) of a normal MEC (76N). Therefore, it was of interest to determine whether established mammary cancer cells showed a down-regulation of its mRNA expression. Indeed, most established breast cancer cell lines showed drastically

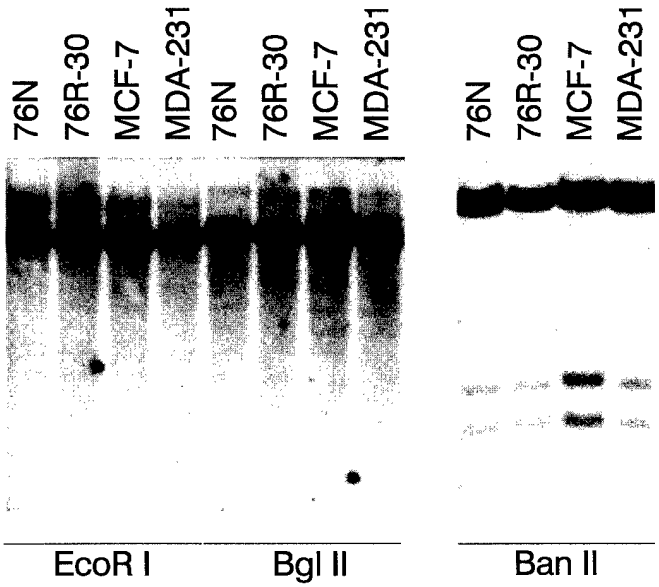


Fig. 9. Southern blot analysis of *NES1* gene. Ten μg genomic DNA from the indicated mammary cell lines were digested with *Eco*RI, *Bgl*II, or *Ban*II restriction enzymes, resolved on a 1% agarose gel, transferred to a nylon membrane, and hybridized with a ^{32}P-labeled NES1 cDNA probe corresponding to nucleotides 1–1069. All cell lines show a similar pattern of bands of almost equal intensity.

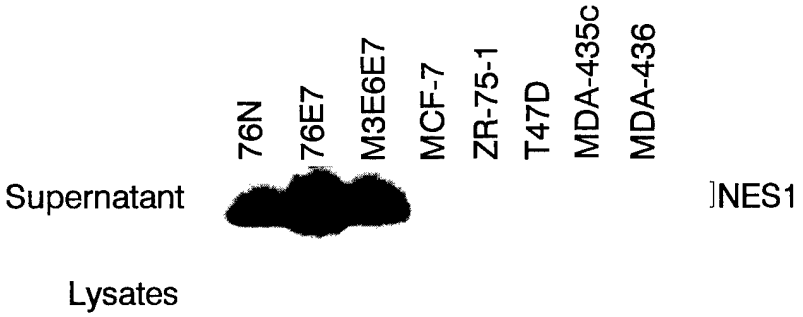


Fig. 10. Western blot analysis of NES1 protein. Aliquots of concentrated culture supernatant or cell lysates derived from number of indicated cell lines were resolved by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Membranes were immunoblotted with anti-NES1 antiserum followed by goat antirabbit IgG conjugated to horseradish peroxidase. Detection was by enhanced chemiluminescence.

decreased or absent NES1 expression. Interestingly, the levels or activities of several proteases show increases during malignant progression (33). In breast cancer cells, the increased levels of cathepsin D, plasminogen activator, and stromolysin (matrix metalloprotease III) have been shown to correlate with tumorigenic progression (34–36). A positive association with tumor aggressiveness has also been noted for a number of proteases, such as urokinase (37, 38), and protease inhibitors block tumor cell invasion (39). Contrary to these examples, however, NES1 expression negatively correlated with tumorigenic progression, suggesting that the products of NES1 proteolytic activity are likely to be involved in negative regulation of growth and/or tumorigenic behavior of mammary epithelial cells. Such a negative growth regulatory role of proteases is not unprecedented. For example, the cysteine protease interleukin 1β-converting enzyme, a homologue of the *Caenorhabditis elegans* death gene *ced-3*, is known to participate in apoptosis of cells (40–43). Interestingly, inhibitors of interleukin 1β-converting enzyme prevented the apoptosis of mammary epithelial cells that was induced by lack of extracellular matrix-derived signals (41). Recent studies using subtractive hybridization and differential display methods have identified other genes, such as a serpin homologue designated maspin and protease inhibitor elafin, the mRNA expression of which were decreased in tumor cells compared with normal mammary epithelial cells (44, 45). These genes have been designated class II tumor suppressor genes, to distinguish them from classical tumor suppressors, which are either mutated or deleted at the DNA level (46). Based on the loss of NES1 expression at the RNA level, this gene may also qualify for categorization as a class II tumor suppressor gene. However, this protein does not have any structural or predicted functional homology to other proteins, such as maspin or elafin, that have been grouped in this category.

Although NES1 has thus far been characterized primarily in the context of mammary epithelial cells, it is expressed in a number of other tissues, although not in all organs, suggesting a potentially organ-specific biological role for this protein. Interestingly, a smaller transcript was observed in the pancreas. The basis of this phenomenon remains to be elucidated. Furthermore, the mRNA expression was observed in epithelial cells but not in fibroblasts, indicating that in the context of a single organ, the function of NES1 may be restricted to certain cell types. However, definitive identification and characterization of NES1 targets will be required to fully unravel the mode of NES1 function. The *in vitro* studies of NES1 expression included mammary epithelial cells belonging to the "luminal" type (milk cells) as well as mammoplasty-derived normal epithelial cells, which may include both luminal and myoepithelial cells (14, 48, 49). However, direct studies of mammary tissues by *in situ* hybridization and immunohistochemistry will be required to directly ascertain the relative expression of NES1 in different mammary epithelial cell types.

We have cloned a novel putative serine protease gene, *NES1*, the expression of which is regulated during tumor progression of mammary epithelial cells. Based on the loss of its expression in breast cancer cells and its sequence homology to known serine proteases, we suggest that *NES1* may regulate the activity of growth stimulatory or inhibitory factors in MECs. Further studies of the protein encoded by this gene are likely to provide insights into mammary cell growth control and tumorigenesis.

ACKNOWLEDGMENTS

We thank Drs. Qingshen Gao for suggestions throughout the study and Hamid Band for the critical reading of the manuscript.

REFERENCES

1. Farber, E. The multistep nature of cancer development. Cancer Res., *44:* 4217–4223, 1984.
2. Sager, R. Tumor suppressor genes: the puzzle and the promise. Science (Washington DC), *246:* 1406–1412, 1989.
3. Tokunaga, M., Land, C. E., Tokuoka, S., Nishimori, I., Soda, M., and Akiba, S. Incidence of female breast cancer among atomic bomb survivors, 1950–1985. Radiat. Res., *138:* 209–223, 1994.
4. Boice, J. D., Jr., Harvey, E. B., Blettner, M., Stovall, M., and Flannery, J. T. Cancer in the contralateral breast after radiotherapy for breast cancer. N. Engl. J. Med., *326:* 781–785, 1992.
5. Boice, J. D., Jr., Preston, D., David, F. G., and Monson, R. R. Frequent chest X-ray fluoroscopy and breast cancer incidence among tuberculosis patients in Massachusetts. Radiat. Res., *125:* 214–222, 1991.
6. Wazer, D. E., Chu, Q., Liu, X-L., Gao, Q., Safaii, H., and Band, V. Loss of p53 protein during radiation transformation of primary human mammary epithelial cells. Mol. Cell. Biol., *14:* 2468–2478, 1994.
7. Control of enzymatic acitivity. *In:* L. Stryer (ed.), Biochemistry, Ed. 3, pp. 226–259. New York: W. H. Freeman and Co., 1988.
8. Wang, M-H., Gonias, S. L., Skeel, A., Wolf, B. B., Yoshimura, T., and Leonard, E. J. Proteolytic activation of single-chain precursor macrophage-stimulating protein by nerve growth factor-γ and epidermal growth factor-binding protein, members of the kallikrein family. J. Biol. Chem., *269:* 13806–13810, 1994.
9. Wolf, B. B., Vasudevan, J., Henkin, J., and Gonias, S. L. Nerve growth factor-γ activates soluble and receptor-bound single chain urokinase-type plasminogen activator. J. Biol. Chem., *268:* 16327–16331, 1993.
10. Isackson, P. J., Ullrich, A., and Bradshaw, R. A. Mouse 7S nerve growth factor: complete sequence of a cDNA coding for the α-subunit precursor and its relationship to serine proteases. Biochemistry, *23:* 5997–6002, 1984.
11. Degen, S. J. F., Rajput, B., and Reich, E. The human tissue plasminogen activator gene. J. Biol. Chem., *261:* 6972–6980, 1986.
12. Naka, D., Ishii, T., Yoshiyama, Y., Miyazawa, K., Hara, H., Hishida, T., and Kitamura, N. Activation of hepatocyte growth factor by proteolytic conversion of a single chain form to a heterodimer. J. Biol. Chem., *267:* 20114–20119, 1992.
13. Band, V., and Sager, R. Distinctive traits of normal and tumor-derived human mammary epithelial cells expressed in a medium that supports long-term growth of both cell types. Proc. Natl. Acad. Sci. USA, *86:* 1249–1253, 1989.
14. Wazer, D. E., Liu, X-L., Chu, Q., Gao, Q., and Band, V. Immortalization of distinct human mammary epithelial cell types by human papilloma virus 16 E6 or E7. Proc. Natl. Acad. Sci. USA, *92:* 3687–3691, 1995.
15. Band, V., Zajchowski, D., Swisshelm, K., Trask, D., Kulesa, V., Cohen, C., Connolly, J., and Sager, R. Tumor progression in four mammary epithelial cell lines derived from the same patient. Cancer Res., *50:* 7351–7357, 1990.
16. Palazzolo, M. J., Hamilton, B. A., Ding, D., Martin, C. H., Mead, D. A., Mierendorf, R. C., Raghavan, K. V., Meyerowitz, E. M., and Lipshitz, H. D. Phage λ cDNA cloning vectors for subtractive hybridization, fusion-protein synthesis and Cre-loxP automatic plasmid subcloning. Gene (Amst.), *88:* 25–36, 1990.

17. Bjourson, A. J., Stone, C. E., and Cooper, J. E. Combined subtraction hybridization and polymerase chain reaction amplification procedure for isolation of strain-specific Rhizobium DNA sequences. Appl. Environ. Microbiol., *58:* 2296–2301, 1992.
18. Chien, C-T., Bartel, P. L., Sternglanz, R., and Fields, S. The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest. Proc. Natl. Acad. Sci. USA, *88:* 9578–9582, 1991.
19. Mellor, H., Flowers, K. M., Kimball, S. R., and Jefferson, L. S. Cloning and characterization of cDNA encoding rat hemin-sensitive initiation factor-2α (elf-2α) kinase. J. Biol. Chem., *269:* 10201–10204, 1994.
20. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. Isolation of biologically active ribonucleic acid from sources enriched in ribonucleases. Biochemistry, *18:* 5294–5299, 1979.
21. Band, V., Zajchowski, D., Kulesa, V., and Sager, R. Human papilloma virus DNAs immortalize normal human mammary epithelial cells and reduce their growth factor requirements. Proc. Natl. Acad. Sci. USA, *87:* 463–467, 1990.
22. Harlow, E., and Lane, D. Antibodies, A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1988.
23. Kozak, M. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell, *44:* 283–292, 1986.
24. The cell nucleus. *In:* B. Alberts, D. Bray, J. Lewis, M. Raff, K. Roberts, and J. D. Watson, (eds.), Molecular Biology of the Cell, Ed. 2, pp. 528–529. New York: Garland Publishing, Inc., 1989.
25. Keil, B. Trypsin. *In:* P. D. Boyer (ed.), The Enzymes, Vol. 3, Ed. 3, pp. 249–275. New York: Academic Press, 1971.
26. Riegman, P. H. J., Klaassen, P., van der Korput, J. A. G. M., Romijn, J. C., and Trapman, J. Molecular cloning and characterization of novel prostate antigen cDNAs. Biochem. Biophys. Res. Commun., *155:* 181–188, 1988.
27. Laborda, J. 36B4 cDNA used as an estradiol-independent mRNA control is the cDNA for human acidic ribosomal phosphoprotein PO. Nucleic Acids Res., *19:* 3998, 1991.
28. Cheah, K-C., Leong, L. E-C., and Porter, A. G. Site-directed mutagenesis suggests close functional relationship between a human rhinovirus 3C cysteine protease and cellular trypsin-like serine proteases. J. Biol. Chem., *265:* 7180–7187, 1990.
29. Blaber, M., Isackson, P. J., and Bradshaw, R. A. A complete cDNA sequence for the major epidermal growth factor binding protein in the male mouse submandibular gland. Biochemistry, *26:* 6742–6749, 1987.
30. Wolf, D. A., Schulz, P., and Fittler, F. Transcriptional regulation of prostate kallikrein-like genes by androgen. Mol. Endocrinol., *6:* 753–762, 1992.
31. Fukushima, D., Kitamura, N., and Nakanishi, S. Nucleotide sequence of cloned cDNA for human pancreatic kallikrein. Biochemistry, *24:* 8037–8043, 1985.
32. Lee, C. C., and Yamada, K. M. Alternatively spliced juxtamembrane domain of a tyrosine kinase receptor is a multifunctional regulatory site. Deletion alters cellular tyrosine phosphorylation pattern and facilitates binding of phosphatidylinositol-3-OH kinase to the hepatocyte growth factor receptor. J. Biol. Chem., *270:* 507–510, 1995.
33. Liotta, L. A., and Stetler-Stevenson, W. G. Tumor invasion and metastasis: an imbalance of positive and negative regulation. Cancer Res., *51:* 5054–5059, 1991.
34. Winstanley, J. H. R., Leinster, S. J., Cooke, T. G., Westley, B. R., Platt-Higgins, A. M., and Rudland, P. S. Prognostic significance of cathepsin-D in patients with breast cancer. Br. J. Cancer., *67:* 767–772, 1992.
35. Spyratos, F., Martin, P-M., Hacene, K., Romain, S., Andrieu, C., Ferrero-Poüs, M., Deytieux, S., Doussal, V. L., Tubiana-Hulin, M., and Brunet, M. Multiparametric prognostic evaluation of biological factors in primary breast cancer. J. Natl. Cancer Inst., *84:* 1266–1272, 1992.
36. Basset, P., Bellocq, J. P., Wolf, C., Stoll, I., Hutin, P., Limacher, J. M., Podhajcer, O. L., Chenard, M. P., Rio, M. C., and Chambon, P. A novel metalloproteinase gene specifically expressed in stromal cells of breast carcinomas. Nature (Lond.), *348:* 699–704, 1990.
37. Liotta, L. A. Cancer cell invasion and metastasis. The most life-threatening aspect of cancer is the undetected spread of tumor cells throughout the body. Improved understanding of how these cells invade tissues is leading to new treatments. Sci. Am., *266:* 54–63, 1992.
38. Blasi, F. Urokinase and urokinase receptors: a paracrine/autocrine system regulating cell migration and invasiveness. BioEssays, *15:* 105–111, 1993.
39. Stetler-Stevenson, W. G., Liotta, L. A., and Kleiner, D. E., Jr. Extracellular matrix 6: role of matrix metallo-proteinases in tumor invasion and metastasis. FASEB J., *7:* 1434–1441, 1993.
40. Enari, M., Hug, H., and Nagata, S. Involvement of an ICE-like protease in Fas-mediated apoptosis. Nature (Lond.), *375:* 78–81, 1995.
41. Boudreau, N., Sympson, C. J., Werb, Z., and Bissell, M. J. Suppression of ICE and apoptosis in mammary epithelial cells by extracellular matrix. Science (Washington DC), *267:* 891–893, 1995.
42. Hentgartner, M. O., and Horvitz, H. R. *C. elegans* cell survival gene *ced-9* encodes a functional homolog of the mammalian proto-oncogene *bcl-2*. Cell, *76:* 665–676, 1994.
43. Yuan, J., Shaham, S., Ledoux, S., Ellis, H. M., and Horvitz, H. R. The *C. elegans* cell death gene *ced-3* encodes a protein similar to mammalian interleukin-1B-converting enzyme. Cell, *75:* 641–652, 1993.
44. Zou, Z., Anisowicz, A., Hendrix, M. J., Thor, A., Neveu, M., Sheng, S., Rafidi, K., Seftor, E., and Sager, R. Maspin, a serpin with tumor-suppressing activity in human mammary epithelial cells. Science (Washington DC), *263:* 526–529, 1994.
45. Zhang, M., Zou, Z., Maass, N., and Sager, R. Differential expression of elafin in human mammary epithelial cells and carcinomas is regulated at the transcriptional level. Cancer Res., *55:* 2537–2541, 1995.
46. Lee, S. W., Tomasetto, C., and Sager, R. Positive selection of candidate tumor-suppressor genes by subtractive hybridization. Proc. Natl. Acad. Sci. USA, *88:* 2825–2829, 1991.
47. Band, H., Hochstenbach, F., McLean, J., Hata, S., Krangel, M. S., and Brenner, M. B. Immunochemical proof that a novel rearranging gene encodes the T cell receptor δ subunit. Science (Washington DC), *238:* 682–684, 1987.
48. Ronnov-Jessen, L., Petersen, O. W., and Bissell, M. J. Cellular changes involved in conversion of normal to malignant breast: importance of the stromal reaction. Physiol. Rev., *76:* 69–125, 1996.
49. Bartek, J., Bartkova, J., Kyprianou, N., Lalani, E-N., Staskova, Z., Shearer, M., Chang, S., and Taylor-papadimitriou, J. Efficient immortalization of luminal epithelial cells from human mammary gland by introduction of simian virus 40 large tumor antigen with a recombinant retrovirus. Proc. Natl. Acad. Sci. USA, *88:* 3520–3524, 1991.

[CANCER RESEARCH 58, 4782–4786, November 1, 1998]

Advances in Brief

The Role for NES1 Serine Protease as a Novel Tumor Suppressor[1]

Jaya Goyal, Karen M. Smith, Janet M. Cowan, David E. Wazer, Sam W. Lee, and Vimla Band[2]

Departments of Radiation Oncology [J. G., D. E. W., V. B.], and Pediatrics [J. M. C.], New England Medical Center, and Department of Biochemistry [V. B.], and Genetics Program [K. M. S., V. B.], Tufts University School of Medicine, Boston, Massachusetts 02111, and Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02115 [S. W. L.]

Abstract

Previously (Liu *et al.*, Cancer Res., *56:* 3371–3379, 1996), we isolated a novel serine protease-like gene—Normal Epithelial Cell Specific-1 (*NES1*)—that is expressed in normal mammary epithelial cells but is down-regulated in most breast cancer cell lines. Here, we demonstrate that stable expression of NES1 in the NES1-negative MDA-MB-231 breast cancer cell line suppressed the oncogenicity as revealed by inhibition of the anchorage-independent growth and tumor formation in nude mice. Fluorescence *in situ* hybridization localized the *NES1* gene to chromosome 19q13.3, a region that contains genes for related proteases (including the prostate-specific antigen) and is rearranged in human cancers. Similar to breast cancer cell lines, prostate cancer cell lines also lacked NES1 mRNA and protein expression. Together, these results strongly suggest a tumor-suppressor role for NES1 in breast and prostate cancer.

Introduction

Carcinomas, the malignant tumors arising from epithelial cells, constitute the majority of human cancers. In nearly all cases, the etiology of these cancers is unknown. Malignant transformation represents a complex multistep process in which genetic changes and environmental factors including radiation, viruses, carcinogens, and dietary components are considered to play a role (1).

To gain insight into biochemical pathways involved in epithelial cell oncogenesis, we and others have used *in vitro* models of epithelial cell transformation. In one such model, we exposed the normal mammary epithelial cell strain 76N to fractionated doses of γ-irradiation *in vitro*, which was similar to a therapeutic regimen used for cancer treatment and which resulted in the tumorigenic conversion of the cells (2). To isolate genes the products of which contributed toward oncogenic transformation in this system and to identify differentially expressed mRNAs, we carried out subtractive hybridization between the normal parental cell strain 76N and its radiation-transformed cell line 76R-30. This strategy resulted in the isolation of a novel putative serine protease, NES1,[3] the mRNA expression of which was observed in 76N cells but was down-regulated in 76R-30 cells (2). Using a panel of normal and tumor mammary epithelial cell lines, we showed that the expression of NES1 mRNA and protein was absent in a majority of breast tumor cell lines (2).

The predicted NES1 polypeptide showed high homology to a number of serine proteases (3), in particular the members of the trypsin family, the kallikrein family, and the family of proteases that activate the kringle domain-containing growth factors (4–6). The kallikrein family includes the PSA that is increased in the serum of prostate cancer patients and serves as a prognostic marker (7, 8). The kringle domain-containing growth factors include human tissue plasminogen activator and human hepatocyte growth factor activator, which have been linked to oncogenesis (5, 6, 9, 10). The former is increased during tumorigenic progression of cells, whereas the latter is a mitogenic growth factor for a known proto-oncogene, c-*met* (hepatocyte growth factor receptor; Ref. 11). The involvement of close homologues of NES1 in oncogenic transformation suggests a potential function for NES1 in cell growth.

Here, we show that the transfection-mediated reconstitution of NES1 expression in a NES1-negative breast tumor cell line, MDA-MB-231, results in suppression of the tumorigenic phenotype both *in vitro* and *in vivo*. The *NES1* gene localized to chromosome 19q13.3 within the same region that contains *PSA* (8). Similar to mammary epithelial cells, NES1 mRNA and protein were expressed in normal and immortal prostatic epithelial cells but not in tumorigenic prostate epithelial cell lines. Together, these data suggest that NES1 plays a tumor suppressor role in breast, prostate, and possibly other epithelial cells.

Materials and Methods

Tissue Sample and Cell lines. Normal prostate tissue was from a prostactomy specimen obtained from the Beth Israel Deaconess Medical Center (Boston). Normal prostate epithelial cells immortalized with the human papilloma virus type 18 (designated as HPV18-I cells) or SV40 large T antigen (SV40-I; obtained from Dr. J. S. Rhim, National Cancer Institute, Frederick, MD; Refs. 12, 13), the ND1 prostate cancer cell line (obtained from Dr. P. Narayan, University of Florida, Gainesville, FL; Ref. 14), and the MDA-MB-231 breast cancer cell line (obtained from Dr. Ruth Lupu, Lawrence Berkeley National Laboratory, Berkeley, CA) have been described (15). PC3, DU-145, and LNCaP prostate cancer cell lines were obtained from American Tissue Type Collection (Manassas, VA). All of the cell lines used in this study were grown in α-MEM with 10% FCS (16).

Transfection. *NES1* cDNA was cloned into pCMV-neo vector and 8 μg of *Hin*dIII linearized plasmid was used for transfection into MDA-MB-231 cells using the calcium phosphate coprecipitation method as described earlier (16). After G418 (1 mg/ml) selection for 2 weeks, single colonies were isolated and subcultured at 1:3 split ratio.

Northern Blotting. Total cellular RNA was isolated from subconfluent monolayer cells using the guanidinium-isothiocynate method. Ten μg of each RNA was resolved on formaldehyde-1.2% agarose gel and was transferred onto a nylon membrane (Hybond-N; Amersham, Arlington Heights, IL). Hybridization was carried out with ^{32}P-labeled *NES1* cDNA probe (nucleotide 651-1072) as described previously (2).

Western Blotting. Culture supernatants from vector- and *NES1*-transfected cells were collected for 24 h after serum deprivation. Fifty μg (breast cells) or 300 μg (prostate cells) of each supernatant [protein quantitation done using a Bicinchoninic acid protein assay reagent kit (Pierce Chemical Co., Rockford, IL)] were separated on a 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Immobilon-P Millipore, Marlborough, MA). The membranes were blocked with TBST (25 mM Tris, 0.15 M NaCl, and 0.1% Tween 20) containing 5% each of nonfat dry milk and BSA, incubated with either rabbit anti-NES1 antibody or monoclonal antibody against PSA (Ab-2, Neomarkers, Fremont, CA), followed by either goat antirabbit IgG or goat anti-

Received 7/23/98; accepted 9/17/98.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by the Department of Defense Breast Cancer Program.

[2] To whom requests for reprints should be addressed, at Department of Radiation Oncology, Box 824, New England Medical Center, 750 Washington Street, Boston, MA 02111. Phone: (617) 636-4776; Fax: (617) 636-6205; E-mail: VBAND@opal.Tufts.Edu.

[3] The abbreviations used are: NES1, normal epithelial specific-1; PSA, prostate-specific antigen; FISH, fluorescence *in situ* hybridization; IGF, insulin-like growth factor; IGFBP-3, insulin-like growth factor-binding protein 3; DAPI, 4′,6-diamidino-2-phenylindole; DAPI-II, DAPI with *P*-phenylenediamine in PBS and glycerol.

mouse IgG-horseradish peroxidase conjugates, respectively. Enhanced chemiluminescence detection was performed according to manufacturer's instructions (Amersham, Arlington Heights, IL).

Anchorage-independent Growth. MDA-MB-231 cells transfected with either vector or *NES1* were plated at 1×10^5/60-mm dish in a top layer of 0.3% agar (Bacto Agar, Difco, Detroit, MI) with a bottom layer of 0.5% agar (both in 1× complete MEM medium). Two ml of complete medium were added on top on every 4th day; plates were examined for clonal growth under a microscope every other day and photographed after 2 weeks.

Tumor Formation in Nude Mice. *Balb/C* nude mice (ages 4–6 weeks) were obtained from The Jackson Laboratory (Bar Harbor, ME) and allowed to adjust to the institutional animal facility for 1 week before the experiment was initiated. Subconfluent cultures of vector or *NES1*-transfected MDA-MB-231 cells were released with trypsin/EDTA (0.025% trypsin and 0.01% EDTA) and resuspended in normal saline. Five $\times 10^5$ cells in a volume of 0.2 ml were injected s.c. into the mammary fat pad area (below the nipple) of five mice per group. Mice were examined on alternate days for the presence of any palpable tumor growth, and tumor sizes were measured. Tumors were allowed to grow for 4 weeks, at which time mice were photographed and killed.

FISH. FISH was performed using a commercial kit following the procedures recommended by the supplier (Oncor, Inc., Gaithersburg, MD). A 140-kb human genomic DNA from bacterial artificial chromosome (Bac) library (obtained through Research Genetics Inc, Huntsville, AL., by screening the library with a *NES1* cDNA probe) was used as a probe for FISH analysis. 300 ng of genomic *NES1* DNA probe was labeled with biotin-14-dCTP (Oncor, Inc) using the Bioprime DNA labeling system (Life Technologies, Grand Island, NY). The time and concentration of DNA were adjusted to produce biotinylated products of 100–500 bp in length as judged by agarose gel electrophoresis. The labeled DNA was purified through a microspin S-200 HR column (Pharmacia Biotechnologies, Piscataway, NJ) and precipitated with 3 M sodium acetate-ethanol in the presence of human COT-1 DNA (human placental DNA from Life Technologies) and salmon sperm DNA to block nonspecific hybridization due to any repetitive human sequences in the probe. The probe was resuspended in Hybrizol VI hybridization solution (Oncor, Inc). Before hybridization, the probe was denatured at 72°C for 10 min., preannealed for 30 min at 37°C, and chilled on ice.

Cytogenetic metaphase chromosome preparations were prepared from human peripheral blood lymphocytes stimulated with phytohemagglutin (17). Before denaturation, the slides were pretreated in 2× SSC [1× = 0.15 M NaCl, 0.015 M sodium citrate (pH 7.0)] for 30 min at 37°C followed by dehydration in an ethanol series (70–95%). Slides were denatured in 70% formamide, 2× SSC (pH 7.0) at 70°C for 2 min, dehydrated in an ice-cold ethanol series (70–95%), and air-dried. The denatured probe was added to the slide, covered with a glass coverslip, and sealed with rubber cement. The probe was hybridized in a moist chamber at 37°C overnight. Immunocytochemical detection of the hybridized probe was achieved using avidin-FITC and antiavidin repetitive amplification steps (Oncor, Inc.). The slides were mounted in antifade medium containing DAPI-II (Vysis, Inc., Downers Grove, IL) for counterstaining of the chromosomes. Chromosomes were evaluated under a Zeiss fluorescence microscope equipped with appropriate filters for the visualization of FITC, DAPI, and propidium iodide.

Results

***NES1*-transfected Cells Lack Anchorage Independence as well as Ability To Grow in Nude Mouse.** The pattern of NES1 expression—with high levels in normal cells, reduced levels in radiation-transformed 76R-30, and an essentially complete lack of expression in most mammary tumor cell lines (2)—suggested a potential role of NES1 in tumor suppression. To directly assess whether NES1 can function as a tumor-suppressor protein, we introduced either pCMV-neo vector or *NES1*-pCMVneo plasmids into a breast cancer cell line, MDA-MB-231. The choice of the MDA-MB-231 cell line was based on its lack of NES1 expression and its known ability to grow in an anchorage-independent manner as well as its ability to form tumors when implanted in nude mouse (2, 15). After G418 selection, six independent stable clones each of the vector and the *NES1*-transfected MDA-MB-231 cells were tested for the expression of NES1 mRNA and protein. As shown in Fig. 1*A*, 4 (clones 2, 4, 5, and 6) of 6 *NES1*-transfected MDA-MB-231 clones expressed high levels of NES1 mRNA whereas the remaining two (clones 1 and 3) showed very little or no mRNA expression. When analyzed for NES1 protein secretion, four clones (clones 2, 4, 5, and 6) showed considerably high levels of protein as compared to two other clones (clones 1 and 3; Fig. 1*B*). As expected, none of the vector-transfectants showed any NES1 protein (*Lane 1* and data not shown). On the basis of the protein expression, the three strong positive clones (clones 2, 4, and 5) were used for further analyses to examine the effect of NES1 on tumorigenicity.

Previous studies (15) have demonstrated a direct correlation between the tumorigenic phenotype of cancer cell lines such as MDA-MB-231 and the ability to grow in an anchorage-independent manner and to form tumors when implanted into nude mice. As shown in Fig. 2*A*, all of the three vector-transfected clones formed colonies when grown in soft agar, whereas the clonogenicity of the three *NES1*-transfectants was markedly decreased. The experiment was repeated three times, each in triplicate, with similar results. Thus, overexpression of NES1 in a NES1-negative breast cancer cell line abolished the ability of cells to grow in an anchorage-independent manner.

When 5×10^5 cells of *NES1*-transfectant clone 4 or a vector-transfectant were injected s.c. into the mammary gland area of mice, 5 of 5 vector-transfected mice showed palpable tumors within 8–10 days of injection, and these grew progressively, reaching a 2.0×2.0 cm^2 size by 4 weeks (Fig. 2*B*). The tumor from one vector transfectant-implanted mouse was excised to examine for histopathology and to assess the ability of tumor cells to grow *in vitro*. These experiments demonstrated that the tumor was an adenocarcinoma, as expected

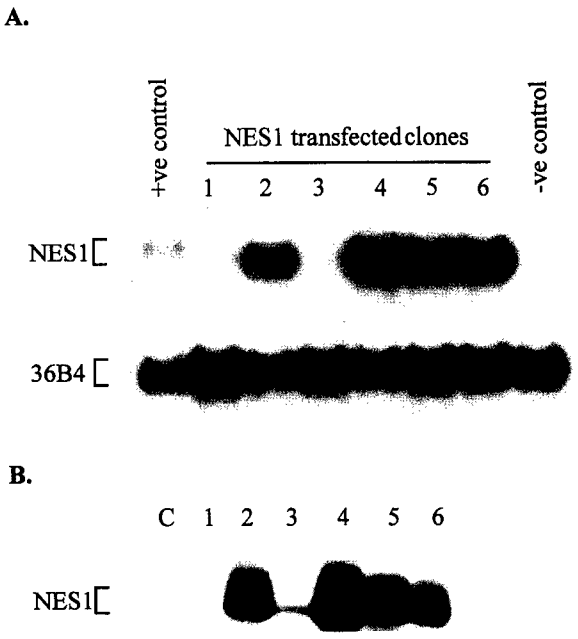


Fig. 1. *A*, Northern blot analysis of NES1 mRNA expression in *NES1*-transfected MDA-MB-231 cells. Nylon membranes with 10 μg of total RNA from different *NES1*-transfected clones (clones 1–6) of MDA-MB-231 cells were hybridized with a ^{32}P-labeled NES1 probe and visualized by autoradiography. Positive (+*ve*) control, normal mammary epithelial 76N cells; negative (-*ve*) control, vector-transfected MDA-MB-231 cells. 36B4 probe was used as a loading control. *B*, Western blot analysis of NES1 protein in *NES1*-transfected MDA-MB-231 cells. Aliquots of culture supernatant derived from different *NES1*-transfected clones (clones 1–6) of MDA-MB-231 cells containing 50 μg of protein were resolved by a SDS-10% PAGE and transferred to polyvinylidene difluoride membrane. Membranes were immunoblotted with an anti-NES1 antiserum followed by goat antirabbit IgG conjugated to horseradish peroxidase. Detection was by enhanced chemiluminescence. Control (*C*), supernatant from vector-transfected MDA-MB-231 cells.

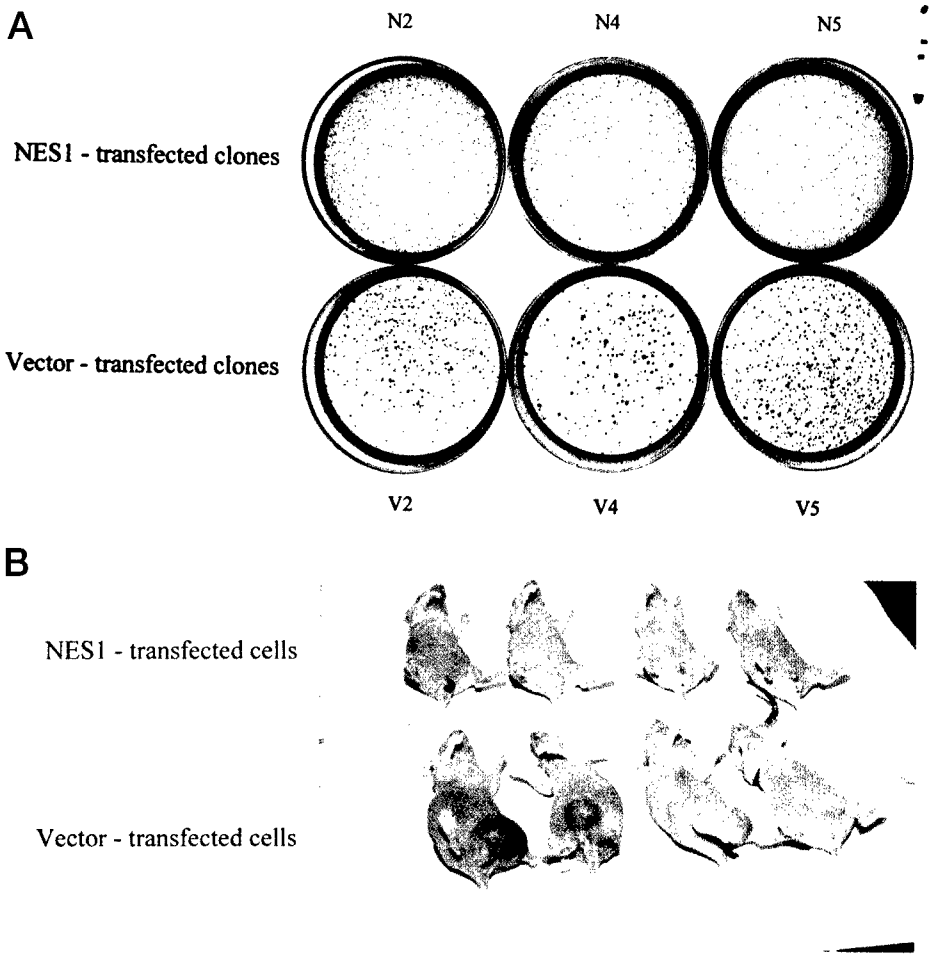


Fig. 2. *A*, anchorage-independent growth of *NES1*-transfected clones. Various clones of vector-transfected (*V2, V4, V5*) or *NES1*-transfected (*N2, N4, N5*) MDA-MB-231 cells (1×10^5/60-mm dish) were plated in soft agar, and colonies were photographed after 2 weeks. *B*, growth of vector- or *NES1*-transfected MDA-MB-231 cells as tumors upon implantation in nude mice. Five $\times$ 10^5 cells of vector or *NES1*-transfected MDA-MB-231 cells were injected s.c. into the mammary fat pad area below the nipple. Tumors were allowed to grow for 4 weeks at which time the mice were photographed and killed.

(15), and these cells proliferated in cell culture (data not shown). In contrast to vector-transfected MDA-MB-231 cells, none of the mice implanted with *NES1*-transfected MDA-MB-231 showed any palpable or visible tumors by 4 weeks. The mice were then killed, and the injected area was examined thoroughly for any nonpalpable tumor growth. However, no tumor growth was observed. Taken together, these data clearly demonstrate the ability of NES1 to suppress tumorigenesis in MDA-MB-231 cells.

Localization of the *NES1* Gene to Chromosome 19q13.3 by FISH Analysis. The potential role of NES1 as a tumor suppressor suggested by the above experiments prompted us to examine the chromosomal localization of the *NES1* gene. For this purpose, we performed pulsed-field gel electrophoresis of *Not*I-digested Bac clone and found that it contained a 140-kb *NES1* DNA insert. Southern blotting of an *Eco*RI-digest of DNA isolated from several single colonies of the Bac clone compared with genomic DNA of 76N normal mammary epithelial cell strain using a *NES1* cDNA probe indicated that the 140-kb genomic clone contained most of the *NES1* gene (data not shown). This 140-kb probe was used for FISH analysis. Thirty-one metaphase spreads were analyzed for chromosomal localization of NES1. In all metaphase spreads, hybridization signals were found on both copies of the 19q (Fig. 3, as indicated by *arrows*). Longer metaphase spreads allowed localization of the *NES1* gene to 19q13.3, and 90% of these spreads had both chromatids stained. Propidium iodide counterstaining to evaluate chromosomal banding confirmed the presence of NES1 on 19q13.3 (data not shown). This analysis localized *NES1* gene to the same region where the gene for prostate cancer-associated serine protease PSA is localized (8). Furthermore, this region is known to undergo loss of heterozygosity in solid tumors including pancreatic carcinomas, astrocytoma, ovarian cancer, and thyroid tumors (17), consistent with a potential tumor-suppressor role of NES1.

NES1 mRNA and Protein Expression Is Down-Regulated in Prostate Cancer Cell Lines. The localization of *NES1* gene to chromosome 19q13.3, where PSA is localized, prompted us to analyze the status of NES1 expression in normal, immortal, and tumor cells derived from the prostate gland. Northern blot analysis revealed that, although NES1 was expressed at high levels in normal tissue from a prostactomy specimen and two HPV-18 and SV40-immortalized normal prostate cell lines, four of four prostate tumor cell lines completely lacked the NES1 mRNA expression (Fig. 4*A*). Importantly, the analysis of NES1 protein in the supernatants of the immortalized prostate cells secreted significant levels of NES1 protein, whereas no protein was detected in the supernatants of prostate tumor cell lines (Fig. 4*B*). Importantly, when these supernatants were tested for PSA levels, a significant level of PSA protein was detected in one of the four (LNCaP) prostate cancer cell lines. These data are consistent with the published reports on the secreted levels of PSA in LNCaP cells (13). Thus, similar to our previous results in breast cells, NES1 mRNA expression is down-regulated during tumorigenic progression in prostate cancer. Taken together, these experiments suggest that NES1 may play a tumor-suppressor role in breast as well as prostate cancer.

Discussion

Our efforts to isolate genes that were differentially expressed in a normal mammary epithelial cell strain 76N and its γ-irradiation-

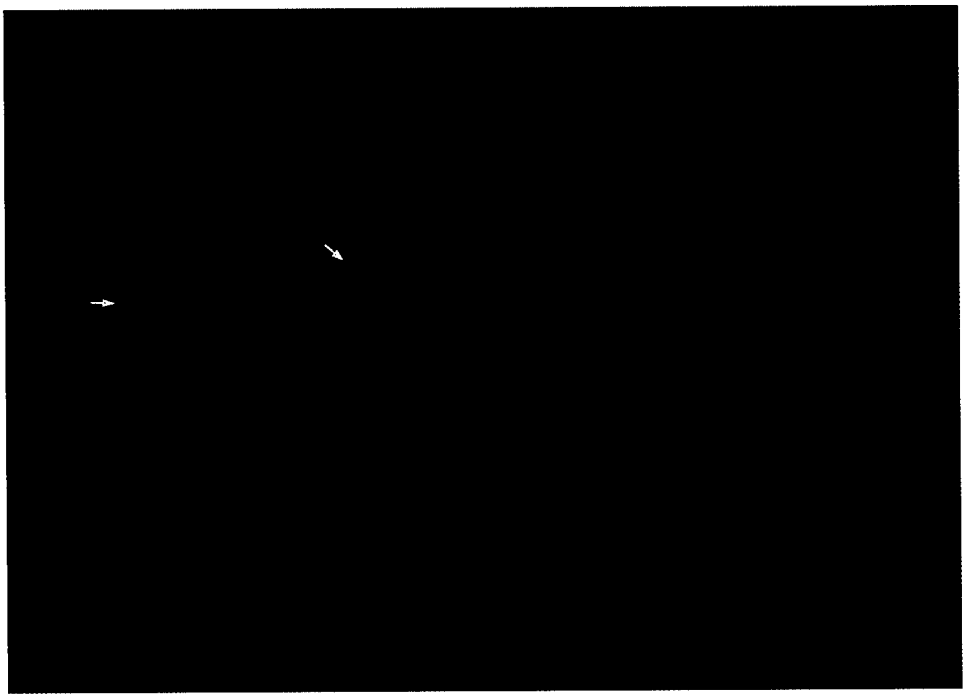


Fig. 3. Localization of *NES1* gene by FISH analysis. FISH analysis was performed using a commercial kit following the procedures recommended by the supplier (Oncor, Inc.) using a biotin-14-dCTP labeled-140-kb *NES1* genomic DNA probe. Cytogenetic metaphase chromosome preparations were from human peripheral blood lymphocytes stimulated with phytohemagglutin. Immunocytochemical detection of the hybridized probe was achieved using avidin-FITC and antiavidin repetitive amplification steps. The slides were mounted in antifade medium containing 4,6-diamino-2-phenylindole DAPI-II for counterstaining of the chromosomes. Chromosomes were evaluated under a Zeiss fluorescence microscope equipped with appropriate filters for the visualization of FITC and DAPI. *Arrows* point to *NES1*-specific hybridization signals in the telomeric region of the long arm of both chromosomes 19 at band q13.3.

transformed derivative, led to the isolation of a novel serine protease whose expression was down-regulated in a majority of breast tumor cell lines (2). Here, we demonstrate that, similar to breast cancers, NES1 expression is also down-regulated at the mRNA level in prostate cancer-derived cell lines, whereas both the mRNA and NES1 proteins are abundantly expressed in immortal prostate epithelial cell lines. Together, the down-regulation of NES1 expression during tumorigenic progression in two different epithelial tumors further supports the likelihood that NES1 functions in regulating cell proliferation, differentiation, or other traits that are deregulated during oncogenesis.

On the basis of its lack of expression in breast cancer cells, we examined the consequence of *NES1* transfection into a NES1-negative breast tumor cell line MDA-MB-231, which allowed an assessment of several aspects of tumorigenic phenotype (2). We demonstrate that *NES1* gene indeed suppressed the tumorigenic phenotype of these cells, as revealed by an essentially complete suppression of anchorage-independent growth in soft agar and growth as implanted tumors in nude mice. This effect was not due to an overall suppression of cell proliferation, inasmuch as vector-transfected cells and *NES1*-transfected cells grow comparably under regular culture conditions.

The potential tumor suppressor role of NES1 is further supported and expanded by our studies using FISH analysis, which localized *NES1* gene on chromosome 19 region *q13.3*. As we have reported earlier, NES1 is highly homologous with serine proteases of the trypsin-like family, kallikrein family, and activators of kringle family. The chromosomal localization of *NES1* gene places NES1 in the kallikrein family of genes that are clustered on chromosome 19q13.3. These results suggest that NES1 may be derived by gene duplication from a common ancestor of the kallikrein family of serine proteases. In humans, this family includes four members: (*a*) human renal/pancreatic kallikrein; (*b*) human glandular kallikrein; (*c*) human PSA; and (*d*) the recently isolated protease M (18). Notably, except for protease M, all of the other members of this family are secreted proteins (2, 3, 18). Interestingly, similar to NES1, protease M also exhibits a reduced expression in breast and prostate tumor cell lines compared with normal cells (18). Although PSA levels are elevated in the serum of prostate cancer patients and their high levels represent a

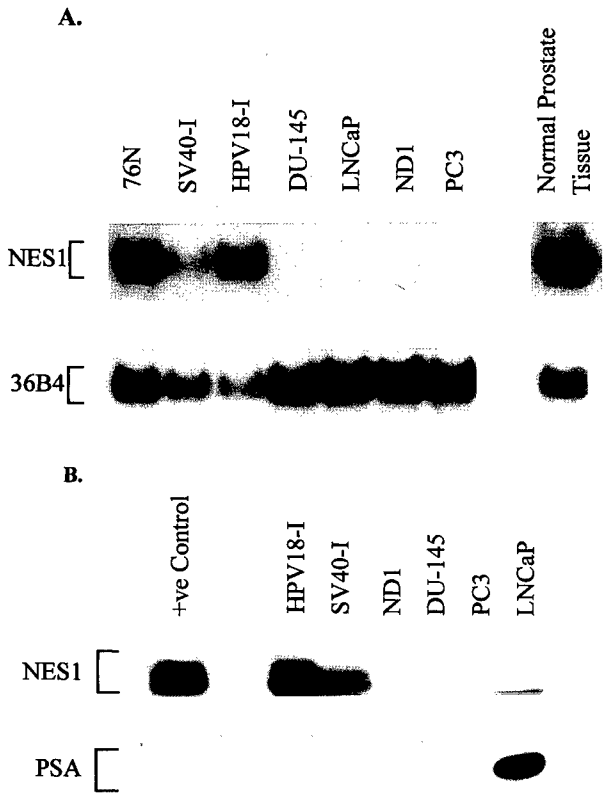


Fig. 4. *A*, Northern blot analysis of NES1 mRNA expression in prostate-derived normal, immortalized, and tumor epithelial cells. Nylon membranes with 10 μg of total RNA from normal prostate tissue, immortalized prostate cell lines SV40-I and HPV18-I, and prostate tumor cell lines DU-145, LNCaP, ND1, and PC3, were hybridized with a ^{32}P-labeled NES1 probe and visualized by autoradiography. 76N normal mammary epithelial cell strain was used as a positive control. 36B4 probe was used as a loading control. *B*, Western blot analysis of NES1 protein in prostate-derived immortalized and tumor epithelial cell lines. Aliquots of culture supernatants derived from different cells (as indicated above) containing 300 μg of protein were resolved by a SDS-10% PAGE and transferred to polyvinylidene difluoride membrane. Membranes were immunoblotted either with an anti-NES1 antiserum (*above panel*) or with an anti-PSA monoclonal antibody followed by goat antirabbit IgG or goat antimouse IgG horseradish peroxidase conjugates, respectively. Detection was by enhanced chemiluminescence. Positive (+*ve*) control, supernatant from *NES1*-transfected MDA-MB-231 cells.

marker of poorer prognosis (7, 8), PSA was reported as marker of better prognosis in breast cancer (19). Thus, two additional members of the kallikrein family that colocalize on 19q13 with NES1 also appear to be closely related to the process of human oncogenesis. Finally, 19q13 is known to be rearranged in a variety of human solid tumors including pancreatic carcinomas, astrocytomas, ovarian cancers, and thyroid tumors (17). Although any direct involvement of NES1 or other kallikrein family member in these rearrangements remains to be analyzed, the chromosomal location of NES1 is fully consistent with its role as a tumor suppressor.

The mechanism of how NES1 induces suppression of the tumorigenic phenotype is currently unknown. Given that NES1 is a secreted serine protease, it is likely that its targets are also extracellular, either components of the extracellular matrix, extracellular growth regulatory molecules or cell surface receptors. It is well documented that normal cellular behavior is regulated by both positive and negative factors. NES1 could mediate its tumor-suppressor role either by generating an inhibitory factor(s) or by terminating the action of an activating factor(s).

Recent studies have shown a role for kallikreins, such as PSA in cleaving IGFBP-3, whereas IGFBP-1,-2,-4, and -6 were not cleaved significantly (as reviewed in Ref. 20). IGFBP-3 has been shown to have IGF1-dependent and IGF1-independent inhibitory effects on cell growth and potential cleavage of IGFBP-3 by PSA may be expected to enhance cell growth, consistent with the correlation of the high levels of PSA and low levels of IGFBP-3 with tumor burden in prostate cancer (20, 21). However, a similar mechanism for NES1 would not account for its tumor suppressor function. Interestingly, whereas a decrease in the levels of IGFBP-3 was observed in the sera of patients with prostate cancer, a significant elevation of IGFBP-2 was noticed (22). Furthermore, patients with high serum levels of PSA also showed elevated levels of IGFBP-2 (22). Interestingly, breast carcinoma cells synthesize a number of IGFBPs, with estrogen receptor-positive and -negative cells secreting different types of IGFBPs (23). Recently, it has been shown that IGFBP-3 predisposes breast cancer cells to program cell death in a non-IGF-dependent manner (24). It is, therefore, possible that NES1 may target one or more IGFBP family members. Additional studies will need to address whether this is the case and whether other targets of NES1 relevant to its tumor-suppressor function exist.

Altogether, our studies support a novel tumor-suppressor function for the serine protease NES1 that is transcriptionally down-regulated during breast and prostate tumor progression. Given this information, it is likely that NES1 may also be involved in a critical aspect of regulating normal epithelial cell growth and/or differentiation.

References

1. Farber, E. The multistep nature of cancer development. Cancer Res., *44:* 4217–4223, 1984.
2. Liu, X-L., Wazer, D. E., Watanabe, K., and Band, V. Identification of a novel serine protease-like gene, the expression of which is down-regulated during breast cancer progression. Cancer Res., *56:* 3371–3379, 1996.
3. Biochemistry. L. Stryer (ed.), 3rd ed., pp 226–259. New York: W. H. Freeman and Company, 1988.
4. Wang, M-H., Gonias, S. L., Skeel, A., Wolf, B. B., Yoshimura, T., and Leonard, E. J. Proteolytic activation of single-chain precursor macrophage-stimulating protein by nerve growth factor-γ and epidermal growth factor-binding protein, members of the kallikrein family. J. Biol. Chem., *269:* 13806–13810, 1994.
5. Degen, S. J. F., Rajput, B., and Reich, E. The human tissue plasminogen activator gene. J. Biol. Chem., *261:* 6972–6980, 1986.
6. Naka, D., Ishii, T., Yoshiyama, Y., Miyazawa, K., Hara, H., Hishida, T., and Kitamura, N. Activation of hepatocyte growth factor by proteolytic conversion of a single chain form to a heterodimer. J. Biol. Chem., *267:* 20114–20119, 1992.
7. Watt, K. W., Lee, P. J., M'Timkulu, T., Chan, W. P., and Loor, R. Human prostate-specific antigen: structural and functional similarity with serine proteases. Proc. Natl. Acad. Sci. USA, *83:* 3166–3170, 1986.
8. Becker, C., and Lilja, H. Individual prostate-specific antigen (PSA) forms as prostate tumor markers. Clin. Chim. Acta, *257:* 117–132, 1997.
9. Dano, K., Andreasen, P. A., Grondahl-Hansen, J., Kristensen, P. L., Nielsen, L. S., and Skriver, L. Plasminogen activators, tissue degradation, and cancer. Adv. Cancer Res., *44:* 139–266, 1985.
10. Lamszus, K., Jin, L., Fuchs, A., Shi, E., Chowdhury, S., Yao, Y., Polverini, P. J., Laterra, J., Goldberg, I. D., and Rosen, E. M. Scatter factor stimulates tumor growth and tumor angiogenesis in human breast cancers in the mammary fat pads of nude mice. Lab. Invest., *76:* 339–353, 1997.
11. Beviglia, L., Matsumoto, K., Lin, C. S., Ziober, B. L., and Kramer, R. H. Expression of the c-met/HGF receptor in human breast carcinoma: correlation with tumor progression. Int. J. Cancer, *74:* 301–309, 1997.
12. Bright, R. K., Vocke, C. D., Emmert-Buck, M. R., Duray, P. H., Solomon, D., Fetsch, P., Rhim, J. S., Linehan, W. M., and Topalian, S. L. Generation and genetic characterization of immortal human prostate epithelial cell lines derived from primary cancer specimens. Cancer Res., *57:* 995–1002, 1997.
13. Webber, M. M., Bello, D., Kleinman, H. K., Wartinger, D. D., Williams, D. E., and Rhim, J. S. Prostate specific antigen and androgen receptor induction and characterization of an immortalized adult human prostatic epithelial cell line. Carcinogenesis (Lond.), *17:* 1641–1646, 1996.
14. Narayan, P., and Dahiya, R. Establishment and characterization of a human primary prostate adenocarcinoma cell line (ND-1). J. Urol., *148:* 1600–1604, 1992.
15. Zugmaier, G., Paik, S., Wilding, G., Knabbe, C., Bano, M., Lupu, R., Deschauer, B., Simpson, S., Dickson, R. B., and Lippman, M. Transforming growth factor β1 induces cachexia and systemic fibrosis without an antitumor effect in nude mice. Cancer Res., *51:* 3590–3594, 1991.
16. Band, V., Zajchowski, D., Kulesa, V., and Sager, R. Human papilloma virus DNAs immortalize normal human mammary epithelial cells and reduce their growth factor requirements. Proc. Natl. Acad. Sci. USA, *87:* 463–467, 1990.
17. Heim, S., and Mitelman, F. (eds.). Cancer Cytogenetics: Chromosomal and Molecular Genetic Aberrations of Tumor Cells. New York: John Wily and Sons, Inc., 1995.
18. Anisowicz, A., Sotiropoulou, G., Stenman, G., Mok, S. C., and Sager, R. A novel protease homolog differentially expressed in breast and ovarian cancer. Mol. Med., *2:* 624–636, 1996.
19. Yu, H., Diamandis, E. P., and Sutherland, D. J. Immunoreactive prostate-specific antigen levels in female and male breast tumors and its association with steroid hormone receptors and patient age. Clin. Biochem. *27:* 75–79, 1994.
20. Cohen, P., Graves, H. C. B., Peehl, D. M., Kamarei, M., Giudice, L. C., and Rosenfeld, R. G. Prostate-specific antigen (PSA) is an insulin-like growth factor binding protein-3 protease found in seminal plasma. J. Clin. Endocrinol. Metab. *75:* 1046–1053, 1992.
21. Rajah, R., Katz, L., Nunn, S., Solberg, P., Beers, T., Cohen, P. Insulin-like growth factor binding protein (IGFBP) proteases: functional regulators of cell growth. Prog. Growth Factor Res., *6:* 273–284, 1995.
22. Ho, P. J., and Baxter, R. C. Insulin-like growth factor-binding protein-2 in patients with prostate carcinoma and benign prostate hyperplasia. Clin. Endocrinol., *46:* 145–154, 1997.
23. Clemmons, D. R., Camacho-Hubner, C., Coronada, E., and Osborne, C. K. Insulin-like growth factor binding protein secretion by breast carcinoma cell lines: correlation with estrogen status. Endocrinology, *127:* 2679–2686, 1990.
24. Gill, Z. P., Perks, C. M., Newcomb, P. V., and Holly, J. M. P. Insulin-like growth factor-binding protein (IGFBP-3) predisposes breast cancer cells to programmed cell death in a non-IGF-dependent manner. J. Biol. Chem., *272:* 25602–25607, 1997.